Biophysical Characterization of a Thermoalkaliphilic Molecular Motor with a High Stepping Torque Gives Insight into Evolutionary ATP Synthase Adaptation*

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Duncan G. G. McMillan†1, Rikiya Watanabe†, Hiroshi Ueno†, Gregory M. Cook§, and Hiroyuki Noji‡1

From the †Department of Applied Chemistry, Graduate School of Engineering, The University of Tokyo, Tokyo 113-8656, Japan and the ‡Department of Microbiology and Immunology, Otago School of Medical Sciences, University of Otago, P. O. Box 56, Dunedin 9054, New Zealand

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F1F0 ATP synthases are bidirectional molecular motors that translocate protons across the cell membrane by either synthesizing or hydrolyzing ATP. Alkaliphilic ATP synthases are highly adapted, performing oxidative phosphorylation at high pH against an inverted pH gradient (acidin/alkaline out). Unlike mesophilic ATP synthases, alkaliphilic enzymes have tightly regulated ATP hydrolysis activity, which can be relieved in the presence of lauryldimethylamine oxide. Here, we characterized the rotary dynamics of the *Caldalkalibacillus thermoarum* TA2.A1 F1 ATPase (TA2F1) with two forms of single molecule analysis, a magnetic bead duplex and a gold nanoparticle. TA2F1 rotated in a counterclockwise direction in both systems, adhering to Michaelis-Menten kinetics with a maximum rotation rate (Vmax) of 112.4 revolutions/s. TA2F1 displayed 120° unitary steps coupled with ATP hydrolysis. Torque measurements revealed the highest torque (52.4 piconewtons) derived from an F1 molecule using fluctuation theorem. The implications of high torque in terms of extreme environment adaptation are discussed.

ATP synthases are membrane-bound rotary molecular motors that are the major enzymes responsible for ATP synthesis in the majority of aerobic life (1, 2). A key feature of the enzyme is the mechanism by which the translocation of protons (or Na+ ions) across the membrane barrier is linked to either synthesizing or hydrolyzing ATP (1–3). Bacterial F1F0-type ATP synthases are two-domain enzymes and are typically composed of eight subunits, α3β3γδεab2c10–15 (4, 5). The soluble F1 domain is composed of the α3β3γ̂δ̂ɛ̂ab2ĉ10–15 subunits in which the α- and β-subunits are alternatively arranged in a hexameric ring-shaped catalytic core. ATP hydrolysis and synthesis occur in the catalytic sites that are formed by each of the β-subunits (4, 6). The core rotary shaft is composed of the γ- and ε-subunits. The γ-subunit penetrates the center of the α3β3 catalytic hexamer at one end and is associated with the α- and c-subunits of the F0 domain at the other end (7). The F0 domain is composed of ab2c10–15 subunits and is membrane-bound (8). The c-subunits form a ring parallel to the membrane (9); however, although stoichiometry is fixed within a species, it is variable between species (10–15). The a-subunit forms a collar around the c-ring and is thought to conduct ions both into and out of the c-ring in a two-channel model (16–19). The c-ring rotates and, together with a-subunit, conducts protons or Na+ ions across the cell membrane (9, 20). The tightly associated γ- and ε-subunits together with the c-ring functionally form the rotor portion of the enzyme, whereas the α3β3δab2 subunits form the stator (4, 5). The coupling of ion translocation through the ac-ring complex to the clockwise rotation of the rotor forces conformational changes within the β-subunits in the α3β3 catalytic hexamer, catalyzing the synthesis of ATP from ADP and inorganic phosphate (21). Conversely, the α3β3 catalytic hexamer can hydrolyze ATP, causing anticlockwise rotation of the γεc10–15 rotor, resulting in the pumping of either protons or Na+ ions into the bulk phase (21–24). It is also noteworthy that ATP hydrolysis is suppressed in aerobic alkaliphilic organisms (25–27).

When the F1 domain is separated from the F0 domain it can be regarded as an ATPase and is useful for the study of the mechanical properties of ATP/ADP-Pi catalysis and enzyme function (28). Indeed, the rotation of F1 ATPases has been visualized using optical microscopy by attaching a probe to the γ- or ε-subunit in the relative area of where the F0 domain would be located had it been attached (23, 28, 29). As this method of study has developed, the type of probe utilized has diversified from actin filaments (23, 30) into gold nanoparticles/nanorods (31, 32), magnetic beads (33, 34), polystyrene beads (35, 36), and fluorescent molecules (37–39). The two most heavily studied bacterial F1 ATPases come from the thermophile *Bacillus* PS3 (TF1) and the mesophile *Escherichia coli* (EF1). Both enzymes are capable of ATP hydrolysis activity, rotating stepwise at an ATP concentration-dependent rate in an anticlockwise direction (23, 40). Because the hexameric α3β3 structure harbors three catalytic sites, the basic step size is 120° coupled to either the consumption or production of one ATP molecule (41). For both TF1 and EF1, these 120° steps have been shown to consist of substeps of 80° and 40° and of 85° and 35° substeps, respectively (35, 40, 42). The 80°/85° substeps are proposed to be tric-
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generated by ATP binding and ADP release and are referred to as ATP-binding (ATP-waiting) dwell (37), whereas the $40^\circ$/$35^\circ$ substeps have been shown to occur after ATP cleavage and release of inorganic phosphate and are referred to as catalytic dwell (43).

Upon catalysis, the generation of torque at the interface between the rotor ($\gamma\varepsilon$ subcomplex) and the stator ($\alpha_{\text{F}1}\beta_{\text{F}1}\delta_{\text{F}1}$ subcomplex) results in the rotation of the F$_1$, by using the chemical free energy from ATP hydrolysis. Due to the interconversion of chemical free energy and electrochemical potential via mechanical rotation, torque is an important factor affecting the conversion efficiency of a molecular rotor. Stepping torque has been estimated for TF$_1$ (41, 44), EF$_1$ (40), the thermophilic *Thermus thermophilus* V$_1$ ATPase (TtV$_1$) (44, 45), and the acidophilic *Enterococcus hirae* V$_1$ ATPase (EhV$_1$) (44), the last of which is proposed to act solely as a V$_1$V$_0$-type ATPase (24, 44, 46). Interestingly, of the F$_1$-type enzymes described here, the torque appears to be higher in the thermophilic TF$_1$ and, according to some reports, lower in EF$_1$. This implies that adaptation to temperature might result in an enzyme with higher torque. In agreement with this, of the V$_1$-type enzymes, the TtV$_1$ torque is greater than that of EhV$_1$; however, the torque difference between TtV$_1$ and EhV$_1$ is much greater than that between TF$_1$ and EF$_1$, suggesting that function may also have a role in defining torque, i.e. ATP synthesis versus hydrolysis (see Table 1).

In this study, we examine the F$_1$ ATPase from the thermoalkaliphilic *Caldalkalibacillus thermarum* TA2.A1. Alkaliphile ATP synthases are highly adapted, performing oxidative phosphorylation at high pH against an inverted pH gradient (acid=$\varepsilon$/alkaline=$\alpha$), thereby challenging Mitchell’s chemiosmotic model (25, 27, 47–49). The environmental pressure of an alkaline environment, together with increased membrane permeability to ions at high temperature (50), results in a severe pressure to conserve energy. Unlike the ATP synthases/hydrolases described here, alkaliphilic enzymes have tightly regulated ATP hydrolysis activity (25–27), which can be relieved in the presence of LDAO$^3$ (26, 51). In addition to this, both the $\gamma$- and $\varepsilon$-subunits of the *C. thermarum* TA2.A1 ATP synthase (TA2F$_1$ F$_0$) are proposed to have regulatory roles in ATP hydrolysis (51, 53). Because this enzyme is the antithesis of the EhV$_1$V$_0$ (i.e. it only performs ATP synthesis (26)), it was of significant interest to examine both catalytic mechanism and torque. In addition, due to the thermophilic origin of TA2F$_1$, we are also able to directly compare the effect of alkaliphily on torque. Here, we characterized the rotary dynamics of TA2F$_1$ with two forms of single molecule analysis, a magnetic bead and a gold nanoparticle.

$^3$The abbreviations used are: LDAO, lauryldimethylamine oxide; TA2F$_1$, *C. thermarum* TA2.A1 F$_1$ ATPase; TF$_1$, thermophilic *Bacillus PS3* F$_1$ ATPase; EF$_1$, *E. coli* F$_1$ ATPase; TtV$_1$, *Thermus thermophilus* V$_1$ ATPase; EhV$_1$, *E. hirae* V$_1$ ATPase; pNnm, piconewton nanometer; NTA, nitrilotriacetic acid; fps, frames per second; ATP-$\gamma$S, adenosine 5'-O-(thiotriphosphate); FT, fluctuation theorem; biotin-PEAC$_2$-maleimide, N-6-(biotinylamino)hexanoyl-N'$\text{-}$(2-N-maleimido)ethylpiperazone.

Results

Expression of TA2F$_2$ and TA2F$_2\gamma 2c$ in *E. coli*—To examine rotary dynamics in F$_1$ ATPases, the enzyme must be capable of being labeled with a trackable probe such as a 200-nm magnetic bead duplex or a 40-nm gold nanoparticle. For other enzymes such as the TF$_1$ ATPase (23, 31, 34, 42, 54), this was achieved by the introduction of two cysteine residues in the $\gamma$-subunit. The F$_1$ portion of the ATP operon from *C. thermarum* TA2.A1, coding for TA2F$_2$, $\alpha_{\text{F}2}\beta_{\text{F}2}\gamma_{\text{F}2}\delta_{\text{F}2}$, with a hexahistidine tag at the N terminus of the $\beta$-subunit, and the same enzyme including two cysteines introduced at positions 107 and 210 in the $\gamma$-subunit (TA2F$_2\gamma 2c$; Fig. 1A) were cloned into the expression plasmid pTrc99A and overexpressed in the unc deletion mutant *E. coli* DK8. TA2F$_2$ and TA2F$_2\gamma 2c$ were extracted from *E. coli* strain DK8 cytoplasmic fractions and purified via a three-step purification procedure. SDS-PAGE analysis of the purified recombinant mutant enzyme (TA2F$_2\gamma 2c$) revealed five subunits (viz. $\alpha$, $\beta$, $\gamma$, $\delta$, and $\varepsilon$) that were identical to those of the native F$_1$ purified from the overexpressed WT TA2F$_2$, previously by Keis et al. (51) (Fig. 1B, lane 2). Postpurification, TA2F$_2\gamma 2c$ was specifically biotin-modified via the cysteine residues at positions 107 and 210 in the $\gamma$-subunit for attachment of rotation probes (Fig. 1B, lane 1). This was confirmed by Western blotting (Fig. 1B, lane 2), revealing that biotin was specifically incorporated into the $\gamma$-subunit of TA2F$_2\gamma 2c$.

ATP Hydrolysis of TA2F$_2\gamma 2c$-Biotin—A feature of the TA2F$_2$ enzyme is its specific blockage in ATP hydrolysis activity and that this activity can be stimulated up to 30-fold with 0.05% LDAO (26, 51). Like the recombinantly expressed native TA2F$_2$, TA2F$_2\gamma 2c$-biotin was blocked in ATP hydrolysis activity. Neither the WT nor the biotin-modified mutant displayed any measurable ATP hydrolysis activity (Fig. 1, C and D). ATP hydrolysis could be induced in TA2F$_2\gamma 2c$-biotin at both 25 and 45 °C with 0.1% LDAO (Fig. 1, E and F). Per TA2F$_2\gamma 2c$-biotin molecule, this resulted in a maximal specific activity of 124.3 mol/s (30.4 units/mg of protein), 141.6 (34 units/mg of protein), and 166.2 mol/s (Fig. 1B) at 25, 45, and 65 °C, respectively. The $K_m$ for ATP was 0.27 mm at 25 °C, 0.28 mm at 45 °C, and 0.26 mm at 65 °C. The activities reported here are consistent with those observed previously for TA2F$_2$, by Keis et al. (51) (28.5 units/mg of protein at 45 °C). We have further optimized the assay by introduction of 50 mM KCl to optimize pyruvate kinase activity (data not shown). In this optimized assay system, upon addition of ATP, we observe a small sharp drop indicative of a small amount of ADP present in the ATP. This is followed by a flat line indicating that the TA2F$_2$ ATPase has close to zero ATP hydrolysis activity at 25, 45, and 65 °C (see Fig. 1, C–E, upon ATP introduction).

Rotation Velocity Is Dependent on ATP Concentration—Because TA2F$_2$ does not natively catalyze hydrolysis and we sought to examine TA2F$_2$ rotational dynamics with ATP hydrolysis-driven rotation, we required a system capable of activating F$_1$ ATPase activity. Fortunately, we have two methods to achieve this in the form of LDAO and the use of magnetic beads with magnetic tweezers, the latter of which is well documented to aid in relieving the TF$_1$ ATPase inhibited states (34, 55, 56). Although the use of magnetic beads is a powerful tool, to exam-
negligible, allowing the observation of unloaded rotation of TA2F1 to be observed provided the bead is unobstructed and free to rotate in the assay solution (42). Considering our low flow chamber volume and requirement for accessibility to the chamber volume, assays at 65 °C present a significant technical problem. However, because ATP hydrolysis at 25 °C is 73% of that possible at 65 °C (see Fig. 1E), we considered it appropriate to conduct all rotation experiments forthwith at 25 °C. To observe single enzyme rotation, TA2F1γ2c-biotin was immobilized on an NTA-modified glass surface in a flow chamber using hexahistidine tags at the N terminus of each β-subunit, and a 40-nm streptavidin-functionalized gold nanoparticle was attached at the top of the γ-subunit as a rotation probe (Fig. 2A). Initial experiments did not include an ATP regeneration system or LDAO. These experiments revealed very few rotating molecules, each of which only performed two to five revolutions before lapsing into an inactive state. Henceforth, we performed rotation studies using TA2F1γ2c using a 40-nm gold
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**FIGURE 3. Rotation of TA2F1 reveals three distinct dwell states separated by 120°.** A–C, representative rotation traces recorded using gold nanoparticle as a rotation probe at a variety of ATP concentrations: 2 mM (A), 200 μM (B), and 20 μM (C). Insets from A–C show the x-y trajectories of the centroid of the rotating gold nanoparticle from the respective traces. D–F are distributions of rotary angles shown in A–C. G–I are distributions of duration times of dwells at each 120° step composed of dwells from 15 molecules from rotation traces analyzed collectively at the same ATP concentrations as in A–C. 1, 2 and 3 are the three dwell positions separated by 120°. The bin width across all plots was 0.5 ms, and the step composed of dwells from 15 molecules from rotation traces analyzed collectively at the same ATP concentrations as in A–C. The red curves show the fitting with a triple exponential model: m1 × (exp(−1/m2 × m0) − exp(−1/m3 × m0) − exp(−1/m4 × m0)); m1 = 150, m2 = 0.002, m3 = 0.0005, and m4 = 0.0005. For G, τ1 = 2.2 ± 0.2 ms, τ2 = 0.42 ± 0.04 ms, and τ3 = 0.42 ± 0.24 ms (n = 1835). For H, τ1 = 2.6 ± 0.3 ms, τ2 = 0.32 ± 0.04 ms, and τ3 = 0.98 ± 0.27 ms (n = 1500). For I, τ1 = 6.2 ± 0.9 ms, τ2 = 0.68 ± 0.07 ms, and τ3 = 0.7 ± 0.18 ms (n = 1500). Fitting was conducted with KaleidaGraph version 4.5.

A nanoparticle system in the presence of 0.1% LDAO and an ATP regeneration system. Rotation was observed at a variety of ATP concentrations using total internal reflection dark field microscopy (31) recorded at a rate of 10,000 frames per second (fps). In the presence of LDAO, TA2F1 rotated counterclockwise for continuous revolutions that decreased in velocity upon decrease in ATP concentration (Fig. 2, B and C). Above 1 mM, rotation rates were essentially constant, and below 200 μM ATP, the rotation rates were proportional to ATP concentration. This suggests that ATP binding may be rate-limiting at ATP concentrations below 200 μM. Rotation velocities at various ATP concentrations collectively followed Michaelis-Menten kinetics with a \( V_{\text{max}} \) of 112.4 ± 4.3 revolutions s\(^{-1}\) at 2 mM ATP and an apparent \( K_m \) of 46.85 ± 3.69 μM (Fig. 2C). The second-order rate constant (\( K_{\text{cat}} \) of ATP!) was determined using \( 3 \times V_{\text{max}}/K_m \) derived from Fig. 2C and was estimated to be \( (7.20 ± 0.1) \times 10^6 \text{M}^{-1} \text{s}^{-1} \) assuming that three ATP molecules are hydrolyzed per revolution of the gold nanoparticle attached to the γ-subunit.

**Stepping Behavior and Dwell Time Analysis of TA2F1.**—A feature of F1 ATP synthases is the 3-fold symmetry of the catalytic domain of the enzyme. Typical examples of rotation at ATP concentrations of 2 mM, 200 μM, and 20 μM ATP are shown in Fig. 3, A–F. At almost all ATP concentrations, it was possible to observe clear 120° stepwise rotation of TA2F1 under both \( V_{\text{max}} \) and low ATP conditions as shown in the x-y trajectories and distribution of rotary angles (Fig. 3, A–C, insets, and D–F). Under \( V_{\text{max}} \) conditions, each 120° step was completed within 0.2 ms; however, this time period was highly dependent on ATP concentration, underscoring that rotation rate is directly related to the duration of the intervening 120° pauses. At 2 mM ATP, considerably above the \( K_m \) of 46.85 ± 3.69 μM, these pauses are proposed to represent catalytic dwells. To obtain the time constants and kinetic parameters, the duration of these...
pauses were examined. Unambiguous analysis was achieved by examination of the first 30 s of traces from 10 molecules distributed over 2 orders magnitude of substrate concentration. These were modeled using a three-consecutive reaction scheme representing ATP binding, hydrolysis, and ADP/Pi release. All distributions of the durations showed a convex shape (Fig. 3, G–I). The average time constants at 2 mM, 200 μM, and 20 μM ATP (Fig. 3, G–I) revealed three time constants that were (i) 2.2 ± 0.2, 2.6 ± 0.3, and 6.2 ± 0.9 ms, respectively; (ii) 0.42 ± 0.04, 0.32 ± 0.04, and 0.68 ± 0.07 ms, respectively; and (iii) 0.42 ± 0.24, 0.78 ± 0.27, and 0.7 ± 0.18 ms, respectively. These time constants correspond to (i) ATP-hydrolysis, (ii) ATP binding, and (iii) ADP/phosphate release. It is unclear from this analysis what time constant corresponds to which reaction step. The second-order rate constant \(K_{\text{m}}[\text{ATP}]\) determined from the distribution of the duration of the ATP-waiting dwells was estimated to be \((8.3 ± 0.2) \times 10^6 \text{ M}^{-1} \text{s}^{-1}\), consistent with \((7.20 ± 0.1) \times 10^6 \text{ M}^{-1} \text{s}^{-1}\) determined by \(3 \times V_{\text{max}}/K_m\) derived from Fig. 2C. To resolve what time constant corresponds to which reaction step, we examined the catalytic mechanism to see whether TA2F1 displayed the same substep behavior as TF1 (35). This is achieved by closely examining rotational stepping behavior around the \(K_m\) value. Unfortunately, we were unable to observe substepping using the full TA2F1 construct with a gold nanoparticle; however, the 120° steps were clearly broadened at 50 μM ATP \((K_m = 46.85 ± 3.69 \mu\text{M}; \text{data not shown})\).

Rotation of TA2F1 Using ATP and ATPγS with Magnetic Beads Reveals a Six-dwell Rotation Profile—To avoid using LDAO as an activation method, we replaced the gold nanoparticle with magnetic beads, allowing mechanical activation; this is a well documented method to aid in relieving TF1 inhibited states (34, 55, 56). In place of a gold nanoparticle, a ∼200-nm streptavidin-functionalized magnetic bead duplex was attached to the γ-subunit as a rotation probe (Fig. 4A). Initially, rotation was observed at a saturating ATP concentration of 2 mM using phase-contrast microscopy at 30 fps. As with the gold nanoparticle rotation system, in the absence of LDAO or any mechanical stimulation, very few rotating molecules were observed. Each molecule in this system only performed two to five revolutions before lapsing into an inactive state. However, upon a single forced counterclockwise rotation using magnetic tweezers, TA2F1 was capable of ATP hydrolysis (Fig. 4B)-driven rotation at speeds of up to 6.9 revolutions s\(^{-1}\) (Fig. 4, C and D). Rotation was observed at a variety of ATP concentrations that decreased in velocity upon decrease in ATP concentration, obeying Michaelis-Menten kinetics. The maximum rotary velocity measured under \(V_{\text{max}}\) conditions was 6.5 ± 0.4 revolutions s\(^{-1}\) with an apparent ATP \(K_m\) of 2.72 ± 0.45 μM (Fig. 4, C and D). The suppression of rotation velocity and lowering of apparent \(K_m\) are a typical feature of using a large, high viscosity probe for rotation observation (31, 33, 44). The second-order rate constant \(K_{\text{m}}[\text{ATP}]\) was determined using \(3 \times V_{\text{max}}/K_m\) derived from Fig. 4D and was estimated to be \((9.90 ± 0.1) \times 10^6 \text{ M}^{-1} \text{s}^{-1}\), assuming that three ATP molecules are hydrolyzed per revolution of the γ-subunit. This is very much in agreement with observations with the values for ATP of \((7.20 ± 0.1) \times 10^6 \text{ M}^{-1} \text{s}^{-1}\) in the same assay system using a gold nanoparticle.
However, in the magnetic bead system, these 120° steps were only clearly observed during rotation at very low ATP concentrations (Fig. 5, A–F). Because this is due to the combined effect of camera and probe response times coupled with the high viscous friction of the probe, we changed to using a fast framing camera at 5000 fps to take advantage of the ability to capture stepping. Initially, ATP was used as substrate; however, the 120° steps were simply broadened at 3 μM ATP. In light of this, we attempted to slow the catalytic steps to clarify the stepping behavior. One such method of achieving this is using the slowly hydrolyzable ATP analogue ATPγS (35); however, using this analogue removes the possibility of harnessing the ATP regeneration assay to remove the buildup of Mg-ADP. To limit this unwanted effect, observation times in the rotation assay were limited to 10 min before flushing the flow cell with fresh ATPγS at the desired concentration. Magnetic bead rotation observations at various ATPγS concentrations revealed a maximum rotation velocity of 4.02 ± 0.3 revolutions s⁻¹ under saturating ATPγS conditions (2 mM; Fig. 6A). From the various ATPγS concentrations tested, TA2F₁ has an apparent Kₘ of 5.69 ± 1.33 μM (Fig. 6B). Due to the recording rate being at 5000 fps, discrete 120° steps were observed at most concentrations of ATPγS tested (Fig. 6, C–H). However, at a concentration of 5 μM ATPγS, multistep (six-step) rotary profiles with 50° and 70° substeps were observed (data not shown). Unfortunately, due to the rarity of the events measured and the less than discrete localization, this is a tentative estimate.

**TA2F₁ Torque Is the Highest Estimated Using Fluctuation Theorem**—Because the conservation of energy is key to survival at alkaline pH, the stepping torque of TA2F₁ was measured to examine the power required to turn the rotor to drive ATP hydrolysis. One of the most accurate methods for this type of analysis is by utilizing fluctuation theorem (FT) (44). FT describes entropy production in small non-equilibrium systems, various colloidal particle systems, and more recently biological systems such as RNA hairpins and the F₁ ATPase (44). FT is of particular use as it estimates torque by use of steps isolated from the time courses of rotary angles (Fig. 7A) without the need for an accurate measurement of the frictional drag coefficient of the probe itself. Because this analysis requires perfect 120° stepping motion with close to equal dwell time at each step, we used rotational data of TA2F₁ hydrolyzing ATPγS under Vmax conditions. Ten TA2F₁ rotation traces recorded in the presence of 2 mM ATPγS were analyzed, revealing a rotary torque of 52.4 ± 4 pNnm (Fig. 7, B and C). For comparison with another ATPase under the same conditions and using the same analysis, we also measured the torque for TF₁. In our hands, TF₁ generated a torque of 41.4 ± 2.2 pNnm, which is quite comparable with that reported previously (Table 1), strongly supporting the reliability of the estimated torque value reported.

**Discussion**

Here, we have conducted the first full biophysical interrogation of a thermoalkaliphilic F₁ ATPase in single molecule analysis with simultaneous comparison with biochemical methods. In this study, we comprehensively demonstrate that the TA2F₁ is a unidirectional rotary molecular motor. In previous studies with TA2F₁, the small amount of hydrolysis activity observed was likely due to the presence of αβ₂ incomplete complex (51). However, the fully intact TA2F₁ used here does not natively
hydrolyze ATP. However, in agreement with previous studies (26, 49, 51, 52), TA2F1 hydrolyzes ATP upon either mechanical or chemical induction. In TF1 studies, the requirement for mechanical activation was proposed to be due to inhibition from a classical feedback loop of product inhibition by Mg-ADP (57); however, rotation was always observable without activation mechanically or with LDAO. Furthermore, TF1 biochemically measured ATP hydrolysis activity was only enhanced 3–4-fold with LDAO (58). In contrast, TA2F1 is activated from an inactive state with LDAO or mechanical activation, the mechanisms by which remain to be fully described. Interestingly, the $K_m$ and $K_{on}$ values obtained from bulk-phase and rotation experiments do not agree. The estimation of $K_m$ revealed by gold nanoparticle rotation was 5.95-fold lower than that determined by the bulk-phase biochemical assay, and the estimation of $K_{on}$ reflects this. However, this is unsurprising...
when considering that single molecule analysis specifically selects active molecules, whereas the bulk-phase assay does not, providing an ensemble measurement. The lowering of $K_m$ is a known hallmark for competitive inhibition, suggesting that inactive molecules are likely due to ADP inhibition. In support of this, it is well known that ATP hydrolysis in TF1 is regulated by Mg-ADP inhibition to prevent wasteful ATP consumption (57), a phenomenon yet to be explored in TA2F1. In TF1 studies, the difference between single molecule results and biochemical results has long been attributed to Mg-ADP inhibition. Under single molecule conditions using a 40-nm gold nanoparticle, TF1 has a maximal rotation velocity of 200 revolutions s$^{-1}$; however, in the ATP-regenerating assay under $V_{\text{max}}$ conditions, only 270 ATP/s/molecule were consumed. Because three ATP molecules are consumed per revolution, bulk biochemistry results would imply only 90 revolutions s$^{-1}$ are possible; however, TF1 is 55% faster according to single molecule data (59). In line with this, according to TA2F1 biochemical data, 124.3 ATP/s/molecule are consumed; therefore a maximum velocity of 41.44 revolutions s$^{-1}$ is expected at 25°C. However, TA2F1 single molecule results show a maximal velocity of 112.4 revolutions s$^{-1}$, a 63% faster $V_{\text{max}}$ velocity than that derived from biochemical data. This observation is strikingly close to the biochemical versus single molecule discrepancies reported for the TF1 and EhV1 (24, 44, 59).

At almost all ATP and ATPγS concentrations tested above or below $K_m$, TA2F1 rotation profiles exhibited three dwells separated by 120° (Figs. 3 and 6) when recorded at 5000 fps. Upon using the slowly hydrolyzable substrate ATPγS, six substeps were able to be resolved close to ATPγS $K_m$. This is in close agreement with observations of two other F$_1$ ATPases, TF1 and EF1, that have both been reported to rotate with six dwells per revolution, close to their respective $K_m$ values (35, 40). This observation together with the lack of six-step rotation observed in the EhV1 ATPases (24) and structural differences between F$_1$ and V$_1$ ATPases (60, 61) implies that the interactions between rotor and stator determine the presence or absence of substeps upon enzyme rotation. However, we report the angular spacing of these 50° and 70° substeps conservatively due to insufficient quality and quantity of the molecules observed. In addition, it was observed that in almost all cases, one of the six substeps was masked showing the “six-step profile.”

Conservation of energy is a constant challenge under thermoalkaliphilic conditions. In light of this, it is unsurprising that the stepping torque derived using fluctuation theorem for TA2F1 is very high (52.4 ± 4 pNnm); however, it was unexpected that it would be 20% higher than that for TF1 (44). Although TF1 and other F$_1$ ATPases studied in rotation studies come from organisms that grow at neutral (E. coli and Bacillus PS3) and acidic (E. hirae) pH, the C. thermarum TA2.1 F$_1$ F$_0$ is highly adapted to function at alkaline pH (Fig. 8A) (48, 53, 62). Given the difference in torque between TF1 and TA2F1 (Fig. 8B) despite both enzymes originating from organisms that grow optimally at 65°C and that the internal pH of both organisms is similar at 7.5–8.0 (63, 64), we suggest that the differences in torque across species may be more related to evolutionary adaptation to external pH than temperature (Fig. 8, A and B). It is also noteworthy that both TA2F1 and TF1 torque was measured at 25°C, ruling out temperature as a potential evolutionary pressure on torque. Even if the torque of TF1 measured at

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**TABLE 1**

| Protein | Torque | Known function | Organism growth conditions | Ref. |
|---------|--------|----------------|---------------------------|------|
| TA2F1  | 52 ± 4* | ATP synthase   | pH 9.5/65°C               | This study |
| EhV1   | 20*    | ATP hydrolyase | pH 5.0/25°C               | 41   |
| TV1    | 35*    | ATP Synthase/hydrolase | pH 7.5/65°C               | 45   |
| TF1    | 33 ± 2* | ATP Synthase/hydrolase | pH 7.5/65°C               | 44   |
| EF1    | 39 ± 4* | ATP Synthase/hydrolase | pH 6.5/35°C               | 40   |

* The values were determined by fluctuation theorem.
* The values are the means ± S.D.
* The value was determined from the angular velocity and frictional drag coefficient of the probe.
65 °C was the same as that of EF1 measured at 25 °C, TA2F1 has 13 pNnm greater torque than TF1.

Notwithstanding, a higher torque implies a much stiffer rotor. One of the key features of alkalophilic adaptation is a large c-subunit ring rotor. In the case of C. thermarum TA2.A1, this is a 13-mer (15) with an F1 torque of 52 pNnm in comparison with the E. coli 10-mer (65) with an F1 torque of 30 pNnm (40) and the E. hirae 10-mer (46, 66) with a V1 torque of 20 pNnm (44). This ion coupling ratio is critical in understanding the power of the molecular motor because although velocity and torque are important for determining enzyme efficiency the gearing (i.e. the number of protons coupled to a single revolution event) is also important when considering purpose and efficiency and that C. thermarum TA2.A1 must partially maintain internal pH by synthesis of ATP. However, it is clear that differences in torque cannot be fully ascribed to the number of c-subunits because E. coli and E. hirae both have 10 c-subunits, but E. coli has a 10 pNnm greater torque (40, 44, 65, 66). This strongly suggests that although c-subunit ring size may have a mechanical influence it is the native direction of catalysis (i.e. TA2F1 is a synthase, and EhF1 is a hydrolase) that has the greater mechanical influence. This seems logical given that there is no torque without resistance; the greater the resistance, the greater the torque. Because we are examining TA2F1 in hydrolysis, it is rotating against its native function; hence, one would expect more resistance.

However, in the field of ATPase dynamics, there are several methods by which to estimate torque values for enzymes (Table 1) (28, 40, 44, 67–70). Early EF1 torque measurements were estimated to be 50 pNnm using the drag on the curvature of an actin filament during rotation (67). However, more recently, when rotation of EF1 was assessed using gold nanorods and analysis of torque was conducted using a “power stroke” drag force method, the torque was estimated to be 63 pNnm (68). Clearly, different experimental methods and systems can result in different torque estimations. Here, we use a very conservative method of analysis, “fluctuation theorem,” to directly compare TA2F1, stepping torque with that of TF1, and other enzymes that have been previously analyzed with similar methodology, thus allowing a very reliable comparison between enzymes. Although the stepping torque measured here reflects the shape of rotation potential, future measurement of stall torque will reflect the thermodynamic efficiency. Interestingly, the complete E. hirae V1 V0 ATPase has a higher torque than V1 (44). This could be due to a “tighter” overall structure influenced by V1 V0 contacts and/or the influence of specific subunit interactions, i.e. the observation of reinforced torque by genetic fusion of the F-subunit to the D-subunit in T. thermophilus V1 (70). It remains to be examined whether this is a V-type ATPase feature or applies more generally. Future studies will focus on what features dictate torque, activity of the TA2F1 F0 complete with the c13 ring, the specific role of ADP inhibition, the substep size, and the mechanical roles of the α- and γ-subunits in TA2F1 F0 regulation of hydrolysis.

Experimental Procedures

Bacterial Strains, Plasmids, and Growth Conditions—E. coli DH5α (71) was used for all cloning experiments, and E. coli DK8 (72) lacking the ATP synthase genes encoding the unc operon (Atp) was used to overproduce the TA2F1 (63, 73). For cloning and overexpression of TA2F1 enzyme, the plasmid pTrc99A (Amersham Biosciences) was used. To overproduce F1 ATPases, transformants of E. coli DK8 were routinely grown in 2X YT medium (74) containing 2 g/liter glucose and 100 μg/ml ampicillin at 37 °C with shaking at 200 rpm.

Construction of pTA2F1γ2c (γH107C/γS1210C) and TA2F1 αβγζ Complexes—In a previous study, the genes coding for the C. thermarum TA2.A1 F1 ATPase were cloned into the expression vector pTrc99A, yielding the plasmid pTrcF1 (75). To substitute the native histidine 107 and serine 210 in the γ-subunit of TA2F1 to cysteine residues, double site-directed mutagenesis was carried out using the plasmid pTrcF1 as template with a three-fragment strategy based on the overlap extension PCR method (76). To construct pTA2F1γ2c, an 837-bp PCR product was generated using primers Tg-cysF and Tg-cysMutR2. Lastly, a 331-bp PCR product was generated using primers Tg-cysF and Tg-cysMutR2. The 837- and 331-bp PCR products were then joined by overlap PCR to generate a 1145-bp PCR product using primers Tg-cysMutF2 and Tg-cys-SR. A 331-bp PCR product that overlapped the 3′- and 5′-ends of the 837- and 1492-bp PCR products, respectively, was generated using primers Tg-cysMutF1 and Tg-cysMutR2. The 837- and 331-bp PCR products were then joined by overlap PCR to generate a 1145-bp PCR product using primers Tg-cysF and Tg-cysMutR2. Lastly, the 1145- and 1492-bp PCR products were joined by overlap PCR to generate a 2615-bp fragment using primers Tg-cysF and Tg-cysR and digested with AflII and AfeI, and the resulting 2292-bp fragment was cloned with a 6817-bp fragment from pTrcF1 digested with the same enzymes (75). This new plasmid was designated pDMTA11. All PCRs were conducted with the Phusion high fidelity PCR kit (New England Biosciences) according to the manufacturer’s instructions. Mutant inserts in both plasmids were confirmed to be correct by DNA sequencing, and the recombinant plasmid was transformed into E. coli DK8. Primer sequences and specific details are displayed in Table 2.

Biophysics of a Thermoalkaliphilic F1 ATPase
Biophysics of a Thermoalkaliphilic F₁ ATPase

TABLE 2

| Primer | Sequence (5'–3')* | Description or mutations | Direction |
|--------|------------------|--------------------------|-----------|
| Tg-cysF | ATCTGTTTCTACCGGTGGTGC | 5' external primer upstream from an AIII restriction site | Forward |
| Tg-cysR | AGGAACCGCTGATTTTTCGGG | 3' external primer downstream from an AfeI restriction site | Reverse |
| Tg-cysMutF | GGGAGGCTgtTCGATCTCAAAAGAC | γH107C | Reverse |
| Tg-cysMutR | TGAGCTGAcacGGTTCTCGATC | γH107C | Reverse |
| Tg-cysMutF2 | CGAACCAGCATTGAACTGGTC | γS210C | Forward |
| Tg-cysMutR2 | GACCAGTTCA6GTCCGGTGTC | γS210C | Forward |

* Lowercase letters indicate specific changes in the primer for site-directed mutagenesis.

Expression and Purification of TA2F₁ and TA2F₁γ₂c—The procedure applied was modified as a hybrid of that in McMillan et al. (48) and Keis et al. (51). DK8 harboring plasmid pTrcF1 or pDMTA11 was grown to an A₆₀₀ of 0.4. The culture was induced with 1 mM isopropyl β-D-thiogalactopyranoside, and incubation continued for 4 h. Cells were harvested, washed with precooled resuspension buffer (50 mM Tris (pH 8.0), 5 mM MgCl₂), and resuspended in the same buffer. Phenylmethylsulfonyl fluoride (PMSF) was added to 0.1 mM, and the cells were incubated for 1.5 h at room temperature. Reduced TA2F₁ and incubated for 1.5 h at room temperature. Unreacted biotin-PEAC₅-maleimide was removed by gel filtration through a Nap10 column (GE Healthcare) and buffer-exchanged into 50 mM Tris–HCl (pH 8.0) containing 2 mM MgCl₂, 100 mM NaCl, and 10% glycerol.

SDS-PAGE and Immunoblotting—TA2F₁ and TA2F₁γ₂c preparations were routinely analyzed on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels using the buffer system of Laemmli (77). Polypeptide bands were visualized using Coo massie Brilliant Blue. During immunoblotting, proteins were separated by 12% SDS-PAGE followed by blotting onto a polyvinylidene difluoride (PVDF) membrane. Detection was achieved using a streptavidin-alanine phosphatase conjugate (Roche Applied Science) directed against the biotin-modified γ-subunit of TA2F₁γ₂c. The antibody-specific bands were visualized using the 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate, 4-toluidine detection system (Roche Applied Science).

Protein Assay—Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Sigma) with bovine serum albumin as the standard.

Biochemical Assays—ATP hydrolysis activity was measured using a spectrophotometric ATP-regenerating assay at 25 and 45 °C similar to that used in McMillan et al. (48, 52). The assay mixture contained 100 mM Tris–Cl (pH 8.0), 1.5 mM MgCl₂, 3 mM phosphoenolpyruvate, 50 mM KCl, 0.25 mM NADH, 5 units/ml pyruvate kinase, 6.4 units/ml lactate dehydrogenase, and variable concentrations of sodium ATP (Sigma) or lithium ATP(S) (Roche Applied Science). For measurements at 65 °C, thermophilic enzymes, 10 units/ml pyruvate kinase H11 and 6.4 units/ml lactate dehydrogenase 2, were used (Funakoshi Ltd.). The reaction was initiated by the addition of enzyme or ATP, as indicated, into 1 ml of assay mixture, and the rate of NADH oxidation was monitored continuously at 340 nm using a Jasco V-660 spectrophotometer. Approximately 5–10 μg (as indicated) of recombinant TA2F₁ or TA2F₁γ₂c subunit complexes was used for assays.

Single Molecule Rotation Assays—Single molecule rotation of TA2F₁γ₂c was measured as described previously using either a ~200-nm magnetic bead duplex (high load probe) or a 40-nm gold nanoparticle (low load probe) (33, 44, 55, 78). Briefly, a flow cell was constructed using a Ni²⁺-NTA-modified glass surface and an uncoated cover glass (Matsunami Glass Ltd., Japan), and biotinylated TA2F₁γ₂c subunit complexes were immobilized on the Ni²⁺-NTA surface via a hexahistidine tag introduced at the N terminus of the β-subunit. After 5 min, non-immobilized TA2F₁γ₂c subunit complexes were removed using buffer A (100 mM Tris (pH 8.0), 1.5 mM MgCl₂, 50 mM KCl) followed by a 5-min incubation in 20 mg/ml bovine serum albumin in buffer A to prevent nonspecific interactions of probes with the surface. Either ~0.2-μm high load streptavidin-functionalized magnetic bead duplexes (Seradyn Inc., Indi-
anapolis, IN) or a low load 40-nm streptavidin-functionalized gold nanoparticle (British BioCell International, UK) were subsequently attached to the biotinylated cysteine residues in the TA2F, γ-subunit in buffer A and incubated for 10 min. Non-bound probe was then removed from the flow cell using buffer A. Observation of TA2F, γ2c rotation was initiated after the infusion of either buffer A containing ≥0.2 mM sodium ATP/lithium ATPγS or buffer B (100 mM Tris (pH 8.0), 300 μM MgCl₂, 50 mM KCl) containing <0.2 mM ATP/sodium ATP/lithium ATPγS. In line with previous studies, 3 mM phosphonopyruvate and 2.5 units/ml pyruvate kinase were used to prevent accumulation of ADP in solution; where necessary, 0.1% LDAO was also included in the assay mixture. When using magnetic beads, the rotation of the duplex attached to the TA2F, γ2c γ-subunit was observed using bright field or phase-contrast microscopy and, when necessary, manipulated with home-built magnetic tweezers (33) controlled with custom-made software (Celery, Library) for activation of “stalled” enzyme. Images were recorded on an inverted microscope (IX-71, Olympus, Japan) at 30 fps (FC300M, Takenaka Systems, Japan) for all characterization experiments with the exception of the six-step rotation observation that was recorded using a high speed complementary metal oxide semiconductor camera at 5000 fps (FASTCAM-1024PCI, Photron). When using gold nanoparticle, the rotation of the duplex attached to the TA2F, γ2c γ-subunit was observed using a modified inverted microscope (IX-71) using total internal reflection dark field microscopy (31) with a lens capable of total internal reflection microscopy (APON 60XOTIRF, Olympus) (44). Images were recorded with a high speed complementary metal oxide semiconductor camera at 10,000 fps (FASTCAM-1024PCI).

Torque Measurements—Stepping torque (N) was determined from the rotation trajectories of magnetic duplex beads (~0.2 μm in diameter) attached to TA2F, γ2c by using the FT (44). For continuous rotation of TA2F1, the time evolution of θ(t) can be described by the Langevin equation (Equations 1 and 2).

\[
\Gamma \frac{d\theta}{dt} = N + \xi(t) \tag{1}
\]

\[
[\xi(t)\xi(t')] = 2\Gamma k_B T\delta(t - t') \tag{2}
\]

where \(\Gamma\) is the frictional drag coefficient, \(\xi\) is a random force representing thermal noise, \(k_B\) is the Boltzmann constant, and \(T\) is the room temperature (298 K). \(N\) is torque and is assumed to be a constant value as described previously (44). Observation of rotation of TA2F, γ2c was performed as described above except that observations were recorded at 5000 fps, and 2 mM lithium ATPγS was used (FASTCAM-1024PCI). Only enzymes exhibiting clear and even dwell times of three steps (120°) were used for analysis where the area on each trace used for analysis was the period moving between one 120° step and the next. Because the rotary motor proteins rotate in one direction, the following expression is derived from FT.

\[
\ln \left[ \frac{P(\Delta\theta)}{P(-\Delta\theta)} \right] = N\Delta\theta/k_B T \tag{3}
\]

for the case \(\Delta t = 5\) ms, and then \(\ln[P(\Delta\theta)/P(-\Delta\theta)]\) versus \(\Delta\theta/k_B T\) was plotted. The slope of the resulting plot corresponds to the torque. The torque of each molecule was defined as the maximum value obtained from the FT analysis when using a 4-s moving window with windows starting at 1-ms intervals.

Author Contributions—D. G. G. M. conceived the study, performed the experiments, analyzed the data, and wrote the article. R. W. and H. U. provided critical insight and experimental/analytical knowledge necessary for the magnetic bead and gold rotation assay platforms, respectively. G. M. C. and H. N. provided critical insight into the broader interpretation of results in the view of both physiology and mechanical mechanism.

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