

Power Stroke Angular Velocity Profiles of Archaeal A-ATP synthase versus Thermophilic and Mesophilic F-ATP synthase Molecular Motors

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ABSTRACT

The angular velocities of ATPase-dependent power strokes as a function of the rotational position for the A-type molecular motor A_3B_3DF from the *Methanosarcina mazei* Gö1 A-ATP synthase, and the thermophilic motor $\alpha_3\beta_3\gamma$ from *Geobacillus stearothermophilus* (formerly known as *Bacillus PS3*) F-ATP synthase are resolved at 5 μ s resolution for the first time. Unexpectedly, the angular velocity profile of the A-type was closely similar in the angular positions of accelerations and decelerations to the profiles of the evolutionarily distant F-type motors of thermophilic and mesophilic origins, and differ only in the magnitude of their velocities. *M. mazei* A_3B_3DF power strokes occurred in 120° steps at saturating ATP concentrations like the F-type motors. However, since ATP-binding dwells did not interrupt the 120° steps at limiting ATP, ATP-binding to A_3B_3DF must occur during the catalytic dwell. Elevated concentrations of ADP did not increase dwells occurring 40° after the catalytic dwell. In F-type motors, elevated ADP induces dwells 40° after the catalytic dwell, and slows the overall velocity. The similarities in these power stroke profiles ~~are~~ support a common rotational mechanism for A-type and F-type rotary motors, in which the angular velocity is limited by the rotary position at which ATP-binding occurs, and by the drag imposed on the axle as it rotates within the ring of stator subunits.

The primary source of ATP in archaea such as methanogens is the A_1A_O ATP synthase, which shares several structural features with eukaryotic V_1V_O ATPases, and is evolutionarily distant from the F_1F_O ATP synthases that fulfill this role in other bacteria and eukaryotes (1,2). Metabolism in archaea is coupled to the generation of an H^+ - and/or Na^+ -potential across the membrane, both of which can provide the energy for ATP synthesis by the A_1A_O ATP synthase. Whereas the F_1F_O ATP synthases in prokaryotes and eukaryotes catalyze ATP synthesis at the expense of an electrochemical ion potential, the evolutionarily related V_1V_O ATPases function as ATP-driven ion pumps, and are unable to synthesize ATP under physiological conditions (3-5). Although the cellular function of archaeal ATP synthases is to synthesize ATP powered by a non-equilibrium electrochemical gradient,

they also work as ATP-driven ion pumps to generate an ion gradient under fermentative conditions (6).

The A-ATP synthases are composed of two opposed rotary motors: an integral membrane A_0 complex (ac_x) involved in ion translocation, and a peripheral A_1 complex (A_3B_3CDF) that contains the catalytic sites for a total of nine different subunits (6). Both motors are connected by two peripheral stalks each composed of an EH-heterodimer. These complexes share common features with bacterial F_1F_0 -ATP synthases; (F_1 : $\alpha_3\beta_3\gamma\delta\epsilon$; and F_0 : ab_2c_{9-15}), and V_1V_0 ATPases (V_1 : $A_3B_3CDE_3FG_3H$; and V_0 : $ac_xc'_yc''zde$) (3-6). In each case the motors are connected by a central stalk that serves as an axle, as well as by one (F-ATP synthases) or three (V-ATPases) peripheral stalks. The minimal subunit composition of the F-type rotor during ATPase-driven rotation is the γ -subunit (Figure 1), which is composed of a coiled-coil domain and a globular domain known as the foot (7,8). Recent structures reveal that the A-ATP synthase and V-ATPase subunits D and F assemble in a manner similar to the coiled-coil and foot domains, respectively, of the F-type γ -subunit (8-11). However, in the A-ATP synthase, the orientation of the four-stranded β -sheet of subunit F is tilted $\sim 45^\circ$ relative to the coiled-coil of subunit D as compared to the orientation of the analogous β -sheet in the foot domain relative to the coiled-coil domain of subunit γ .

The drive shaft is surrounded by a ring of three heterodimers, known as the $(\alpha\beta)_3$ -ring in F_1 , and the $(AB)_3$ -ring in A_1 and V_1 . Although each heterodimer comprises a catalytic site, the majority of residues that contribute to nucleotide binding reside on the β -subunit or A-subunit. Each β -subunit and A-subunit has a catalytic domain, and a 'lever' domain. During ATP synthesis and hydrolysis, the central stalk of an F-ATP synthase rotates clockwise and counterclockwise, respectively, relative to the surrounding $(\alpha\beta)_3$ -ring (12-14). Each of the three catalytic sites in the $(\alpha\beta)_3$ -ring work in succession such that, at saturating ATP concentrations, the drive shaft is driven in 120° power strokes that are separated by catalytic dwells when ATP is hydrolyzed (Figure 2). In most available crystal structures, one catalytic site lacks bound nucleotide (β_E), while ATP (β_T), and ADP (β_D) are bound to the other sites. The most prominent conformational change between the empty and nucleotide-

occupied sites is that the lever domain is open in the former, and closed (pushed against the drive shaft) in the latter when nucleotide is bound.

At concentrations that limit the rate of ATP hydrolysis, each 120° power stroke of prokaryotic F₁ complexes is interrupted ~80° prior to the catalytic dwell by an ATP-binding dwell that has a duration proportional to the time required for ATP to bind to the empty catalytic site (8,15-18). However, in the human F₁-ATPase, the ATP-binding dwell occurs 90° prior to the catalytic dwell, and a phosphate release dwell can be induced to occur 25° prior to the catalytic dwell (19). Although the central stalk of the bacterial V-ATPases of *Thermus thermophilus* and *Enterococcus hirae* also rotate in 120° steps separated by catalytic dwells, no additional ATP-binding dwell has been observed at limiting ATP concentrations (20,21). This suggests that, in bacterial V-ATPase A₃B₃DF-complexes, ATP-binding and product release steps occur at the same rotary positions as the catalytic dwell (22).

The angular velocity of the *E. coli* F₁-ATPase power stroke has been determined as a function of rotational position from single-molecule data collected at 200 kHz (equivalent to 200,000 frames per second) with high signal-to-noise using a gold nanorod as a visible probe (8). During the power stroke, the angular velocity of the drive shaft was observed to undergo a sequence of accelerations and decelerations. The slower angular velocity observed with the lower-affinity substrate ITP relative to ATP provided direct evidence that energy to power rotation is at least partly derived from substrate binding. The angular velocity profile during the power stroke was found to correlate with targeted molecular dynamics simulations that used a simple functional form for potential energy coupling of the action of the β-subunit levers on the γ-subunit coiled-coil (23). This correlation provided additional support for the hypothesis that ATP binding-dependent closure of the lever domain applies force to rotate the γ-subunit.

We now report the first high resolution characterization of the ATPase-dependent power strokes of the A₃B₃DF-complex of the *Methanosarcina mazei* Gö1 A-ATP synthase (*MmA*₃B₃DF) as well as those of the α₃β₃γ complex from the thermophilic F-ATP synthase of *Geobacillus stearothermophilus*, formerly known as *Bacillus* PS3 (*Gs*α₃β₃γ). The power strokes of the *MmA*₃B₃DF-complex occurred in 120° degree steps at saturating MgATP concentrations, comparable to those of other prokaryotic V-

ATPases, and F-ATPases (21,22,24). The power stroke angular velocities of the *MmA*₃*B*₃*DF* and *Gs* $\alpha_3\beta_3\gamma$ complexes were both observed to vary during the power stroke as a function of rotational position with a series of accelerations and decelerations. These angular velocity profiles were similar to that reported previously for the mesophilic *E. coli* $\alpha_3\beta_3\gamma$ complex (*Ec* $\alpha_3\beta_3\gamma$) (8), even though the ATP-binding dwell of A-type ATPases was not observed at 80° prior to the catalytic dwell as it occurs in F-type ATPases (15,18). The similarities in these power stroke profiles are consistent with a common mechanism for rotation among the A-type and F-type rotary motors that is largely independent of the position of the ATP-binding dwell. In this mechanism, ATP-binding to the catalytic site induces the closure of the lever domain, and the angular velocity at a given rotational position is limited by the mechanical advantage of the force of the lever acting against the rotor as a cam shaft, and by the drag imposed on the axle as it rotates within the ring of stator subunits.

RESULTS

ATPase activity of the A₃B₃DF-complex from the archaeal M. mazei Gö and its mutants.

The A-ATP synthase complexes of wild type *MmA*₃*B*₃*DF* and its mutants *MmA*₃*B*₃*DF*-M1 and *MmA*₃*B*₃*DF*-M2 were purified as described recently (25). Fig. 3A shows an SDS-gel of the purified complexes with all subunits. The specific ATPase activities of *MmA*₃*B*₃*DF*-complexes were determined in ensemble measurements using the continuous pyruvate kinase/lactic dehydrogenase coupled assay. The activity of the wild type *MmA*₃*B*₃*DF* complex, which contained a His₈-tag at the N terminus of subunit A, was 4.6 $\mu\text{mol ATP min}^{-1}$ (mg protein)⁻¹ (Figure 3B). The His₈-tag served to purify the protein and to assemble the complex on a microscope slide in the single molecule rotation assay. The introduction of a cysteine in subunit D (A71C) for biotinylation and attachment of a neutravidin-coated nanorod in the mutant *MmA*₃*B*₃*DF*-M2 did not significantly affect the ATPase activity. In contrast, removal of all cysteines in the complex except for A71C via amino acid substitutions C36V, C72V, C181A, and C380V in subunit A as well as C67S in subunit B (mutant *MmA*₃*B*₃*DF*-M1) resulted in a decrease in ATPase activity of 54%. Since these cysteines contribute significantly to the hydrolytic activity of the enzyme, and are not accessible for attachment of the

neutravidin-coated gold nanorod, the His₈-tagged A₃B₃D_{A71C}F-complex of the *M. mazei* Gö1 A-ATP synthase was used for single-molecule rotation experiments. For simplicity, this complex will be subsequently referred to as the *MmA*₃B₃DF-complex.

Single-Molecule rotation measurements at a low data acquisition rate.

The ATPase-dependent rotation of the *MmA*₃B₃DF-complex was measured in a single-molecule rotation assay. Each complex was attached to a Ni-NTA functionalized cover slip via its His₈-tags (Figure 4A). In this orientation, a neutravidin-coated gold nanorod (40x76 nm) can dock to the biotinylated C71 of subunit D. Polarized red light scattered from a nanorod attached to a single *MmA*₃B₃DF-complex passed through a polarizing filter prior to collection by a single photon counter. Under these conditions, rotation of the nanorod was observed as sinusoidal changes in red light intensity (26,27).

In Figure 4B, the variation of the intensity of red light scattered from a nanorod attached to a single-molecule of the *MmA*₃B₃DF-complex in the presence of saturating MgATP was observed as a function of the stepped rotation of the polarizer by 10° in 5 s intervals, at a data sampling rate of 1 kHz. This low data acquisition speed was shown to report the positions of the three catalytic dwells of the *Ec*α₃β₃γ-complex (26,27) because the power stroke transitions occur too fast (on a μs time scale) for the detector to capture the vast majority of intermediate rotational positions of the nanorod. Each dwell contributed a peak in the intensity distribution of the histogram at a given set angle of the polarizer. When viewed as a series of histograms of light intensities at each of 36 polarizer angles covering 360°, three off-set sinusoidal curves in scattered light intensities were observed from a nanorod attached to the rotating drive shaft of a single *MmA*₃B₃DF-complex (Figure 4B). Since the dependence of light intensity as a function of nanorod orientation relative to the axis of the polarizer has been shown to be sinusoidal (28), the observation of three off-set sinusoidal curves in the histograms when the axis of rotation was close to the orthonormal position relative to the microscope slide indicates the presence of three dwells per rotation. Thus, these data show that subunit D rotates on a μs time scale in 120°-steps inside the *MmA*₃B₃DF complex separated by dwells that occur on a ms time scale.

Deviation of the axis of rotation from orthonormal has been shown to change the off-set of the three sinusoidal curves (26) as observed here. It is noteworthy that not all peaks have the same height, and some histograms have fewer than three peaks. Differences in peak heights occur at polarizer angles where two sinusoidal curves intersect (26). Otherwise the occurrences in each sinusoidal curve depend on the relative duration of each of the three catalytic dwells. The dominance of one sinusoidal curve may suggest that one catalytic dwell was longer than the others. However, this may also result if the nanorod occasionally hits the surface at that position.

Figure 4D shows a series of histograms of light intensities acquired at 1 kHz at each of 36 polarizer angles covering 360° scattered from a nanorod attached to the γ -subunit drive shaft of the $\alpha_3\beta_3\gamma$ -complex from the thermophilic F-ATP synthase of the purified *Gs* $\alpha_3\beta_3\gamma$ -complex (Figure 4C). In the presence of a saturating concentration of MgATP (1 mM), three off-set sinusoidal curves were observed with *Gs* $\alpha_3\beta_3\gamma$ that were closely similar to those observed with the *Mm*A₃B₃DF-complex. Using small beads as probes, the *Gs* $\alpha_3\beta_3\gamma$ -complex has been shown to rotate counterclockwise in 120° steps separated by catalytic dwells in the presence of saturating MgATP concentrations (15,16,29). The results of Figure 4D provided a positive control that indicated that the three off-set sinusoidal curves observed in the series of histograms using nanorods during ATPase-dependent rotation catalyzed by the *Mm*A₃B₃DF-complex result from 120° rotational events separated by catalytic dwells in a manner similar to that of *Gs* $\alpha_3\beta_3\gamma$.

Single-Molecule rotation measurements at a high data acquisition rate.

Using the same single-molecule rotation assay described above, the angular position of the *Mm*A₃B₃DF-complex as a function of time during a power stroke was resolved by sampling the changes in light intensity scattered from the rotating nanorod at 200 kHz. An example of raw data of scattered light intensity changes collected during a single power stroke as a function of time at saturating ATP concentrations is shown in Figure 5A. Prior to data collection, the polarizing filter was aligned with the nanorod so that the light intensity was near a minimum during one of the catalytic dwells. Consequently, as the power stroke proceeded, the light intensity increased through the maximum, which occurred when the nanorod had rotated 90°, and continued to change in a sinusoidal

manner. The end of the power stroke was evident by appearance of only small fluctuations of the intensity consistent with a catalytic dwell, which occurred at $\sim 400 \mu\text{s}$ in the case of the power stroke in Figure 5. Based on the sinusoidal dependence of light scattered from the nanorod as the result of rotational orientation relative to the polarizer, these data provided direct evidence that $Mm\text{A}_3\text{B}_3\text{DF}$ rotates in 120° power strokes.

Optimization of the Fitting Algorithm.

Martin *et al.* (8) converted scattered light intensity values from a nanorod attached to *E. coli* F_1 -ATPase during a power stroke like intensity change as shown in Figure 5A to angular position using an arcsine function. Due to the intensity fluctuations that occur during the catalytic dwell, it can be difficult to determine the precise point at which any given catalytic dwell ends and the power stroke begins. Although the arcsine function provided a good average fit to the data throughout the duration of the power stroke, we hypothesized that the fit during the first 20° of rotation at near minimal light intensities could be improved using an $\text{arcsine}^{1/2}$ function (Figure 5B) as described in Experimental Procedures.

A comparison of the error inherent in the determining of the rotational position of a nanorod from scattered light intensity by the $\text{arcsine}^{1/2}$ versus the arcsine function used previously (8,28) is shown in Figure 6. In these experiments a nanorod was attached to the γ -subunit of an $E\text{c}\alpha_3\beta_3\gamma$ molecule that was not rotating, and the average scattered light intensity was determined from a series of histograms at polarizer angles every 10° for a total of 120° starting from the minimum intensity. The rotational position of the nanorod was calculated from the intensity using the arcsine or the $\text{arcsine}^{1/2}$ function assuming a perfect fit at the first data point, which was at the intensity minimum (Figure 6A). The total error between the actual rotational positions of the nanorod and the value calculated using the arcsine and $\text{arcsine}^{1/2}$ functions is compared in Figure 6B. In three of the four nanorods examined, the error using the $\text{arcsine}^{1/2}$ function was substantially smaller. In the data set from nanorod-2, the error using arcsine was slightly smaller than that obtained using $\text{arcsine}^{1/2}$, indicating that the former function provided a better fit overall. The lower errors observed with $\text{arcsine}^{1/2}$ resulted primarily

from its ability to fit to the small changes in intensity that occurred during the first 20° and near the maximum (~90°).

The MatLab algorithm (8,26) analyzes power strokes from consecutive data points that begin at scattered light intensities within 5% of the minimum and increase to within 5% of the maximum intensity. The power stroke is clearly distinguished from the catalytic dwell approximately +3° (~one data point) after the minimum. Consequently, the errors derived from arcsine versus arcsine^{1/2} fits were also compared using the same nanorods assuming a perfect fit of the experimental data 3° after the intensity minimum (Figure 6C, D). Under these conditions, smaller errors were observed with the arcsine function for the data from three of the four nanorods, while the error for the data set from nanorod-1 was 12% smaller with arcsine^{1/2}. The smaller errors derived using arcsine were due to the better fit with this function during the first 90°, after which the arcsine^{1/2} function fit more closely to the data. The sum of errors for the data from the four nanorods when the data were analyzed from the minimum using arcsine^{1/2} was 2571. Since this value was smaller than the sum of the errors determined by the other three methods (Figure 6B, D), the arcsine^{1/2} function was used to calculate the average angular velocity as a function of rotational position during the power stroke.

The differences between the two procedures for data derived from the *Ec*α₃β₃γ-complex are compared in Figure 7B. The angular velocity profiles derived from scattered light intensity using the arcsine function and arcsine^{1/2} share most of the same features. These include attaining a steady state velocity at ~52° at the end of Phase-1, and the successive accelerations and decelerations at 85°, ~90°, 94°, and 97°. However, due to the increased precision during the first 15° of the power stroke, the arcsine^{1/2} function resolves a rapid acceleration that peaks at 7° after the catalytic dwell, then decreases to the steady state velocity. In addition, the angular velocities during Phase-2 derived from arcsine^{1/2} are also proportionally smaller than those derived by arcsine.

Comparison of power stroke angular velocity profiles.

The average angular velocity of power strokes between catalytic dwells in the *Mm*A₃B₃DF-complex as a function of rotational position is shown in Figure 7A. The slopes of neighboring data points (Figure 5B) were averaged, and binned for every three degrees of rotation, then plotted versus

time for each power stroke. The average angular velocities of 2319 power strokes from 25 molecules of the *MmA*₃*B*₃*DF*-complex varied as a function of the rotational position from the end of the catalytic dwell. At the beginning of the power stroke, subunits D and F rapidly accelerated to ~ 300 °ms⁻¹ at 7°, then decelerated to a steady-state rate of ~ 200 °ms⁻¹ between 25° and 60°. This was followed by a second acceleration to a maximum of 500 °ms⁻¹ at 88°. During a 3° period at an angular position of $\sim 90^\circ$, there was a steep acceleration to a maximum of 1200 °ms⁻¹. After decelerating to 210 °ms⁻¹ at 94°, the rate accelerated again to a maximum of 245 °ms⁻¹ at 100°, before the power stroke decelerated as it approached next catalytic dwell at 120°.

The average angular velocities of the thermophilic F-ATPase *Gs* $\alpha_3\beta_3\gamma$ -complex were also determined from the power strokes analyzed by gold nanorod data collected at 200 kHz, and were compared to similar data collected from *Ec* $\alpha_3\beta_3\gamma$ -ATPase (Figure 7A). The angular velocities of these thermophilic and mesophilic F-type ATPases varied as a function of the rotational position from the end of the catalytic dwell in a manner that closely paralleled the changes in angular velocity during the power stroke of the *MmA*₃*B*₃*DF*. The differences in the power strokes among these motors were primarily in the angular velocities that each achieved during the acceleration and deceleration phases. During the first 60° of rotation from the catalytic dwell (Phase-1), the angular velocities of the A-type and *Gs*F-type were comparable, but were only about 73% of that of the *Ec* $\alpha_3\beta_3\gamma$. Although the rotation rates of the motors were comparable during the Phase-2 acceleration, the angular velocities of the A-type and *Gs*F-type during the final 30° were 85% of those of *Ec* $\alpha_3\beta_3\gamma$.

Effects of Nucleotides on the power stroke.

Figure 8 compares the effects of ATP concentrations that limit the rate of ATP hydrolysis on the *MmA*₃*B*₃*DF*-complex with those on *Ec* $\alpha_3\beta_3\gamma$. At limiting ATP concentrations, *Ec* $\alpha_3\beta_3\gamma$ exhibited an increase in dwells during Phase-1 of the power stroke (20° to 60° after the catalytic dwell) from that observed at saturating ATP concentrations as reported previously (8). Consistent with ATP-binding dwells, the occurrence of these dwells was maximal after the γ -subunit had rotated $\sim 40^\circ$ from the catalytic dwell (Figure 8A). At this rotational position the occurrence of dwells was two-fold greater than that observed at saturating ATP concentrations.

Since the angular velocity as a function of rotational position is the average of a few thousand power strokes, the occurrence of a dwell at a given rotational position in a significant fraction of the power strokes will register as zero velocity, and thus decrease the average angular velocity at that position. When ATP was limiting, decreases in the average angular velocity were observed for *Ec* $\alpha_3\beta_3\gamma$ during Phase-1 with a maximum decrease of more than 20% at a rotational position of $\sim 40^\circ$ (Figure 8C). This is consistent with the increased occurrence of the ATP-binding dwells.

During ATPase-dependent rotation of the *Mm* A_3B_3DF -complex at 100 μ M MgATP (Figure 8B), no changes in the occurrence of dwells during Phase-1 were observed, even though the rate of ATPase activity at this ATP concentration was less than 30% of the maximal rate (25). The angular velocity of the *Mm* A_3B_3DF -complex during Phase-1 was also the same in the presence of ATP concentrations that limited the rate of ATP hydrolysis as that which saturated the ATPase rate (Figure 8D). The angular velocity during Phase-2 of the angular velocity profile (from 75° to 120°) was about 15% slower when ATP was limiting to ATPase activity, than at saturating ATP concentrations as reported previously (8). However, the occurrence of dwells during this part of the power stroke is rare because the angular velocities are at their highest. Based on these data we conclude that ATP-binding dwells do not interrupt the power strokes of the *Mm* A_3B_3DF -complex. Consequently, ATP-binding dwells in this complex must occur at the same rotary position as the catalytic dwells.

Elevated ADP concentrations (4 mM) were found to induce dwells during the power strokes of *Ec* $\alpha_3\beta_3\gamma$ during Phase-1 of the power stroke with a maximum increase of about two-fold 40° after the catalytic dwell (Figure 9A), as reported previously (8). The presence of additional dwells during this part of the power stroke was also evident as a decrease in the average angular velocity during Phase-1 with a maximum decrease of about 30% (Figure 9C). The average angular velocity of the *Ec* $\alpha_3\beta_3\gamma$ power stroke was also lower during Phase-2 (from 80° to 100°) in the presence of additional ADP, which did not result from an increase in dwells as observed previously (8). In addition, the arcsine^{1/2} analysis, which increases angular resolution during the initial 20° of the power stroke, revealed that high ADP also suppressed the angular velocity in this region that was not due to dwells. In contrast, the *Mm* A_3B_3DF -complex did not exhibit an increase in ADP-binding dwells between 20° and 60°

after the catalytic dwell (Figure 9B), nor was the average angular velocity of the power stroke from this complex affected by the presence of as much as 250 μ M ADP (Figure 9D).

DISCUSSION

The results presented here provide the first high resolution comparison of the power stroke angular velocities as a function of rotational position of the archaeal *MmA*₃B₃DF-complex and the thermophilic *Gs* $\alpha_3\beta_3\gamma$ -complex *versus* that of the mesophilic *Ec* $\alpha_3\beta_3\gamma$ -complex. The angular velocity profiles of these rotary motors are closely similar, and exhibit a series of accelerations and decelerations that occur at nearly the same rotary positions. The drag imposed by a 40x76 nm gold nanorod attached to *Ec* $\alpha_3\beta_3\gamma$ was determined not to be rate-limiting to ATPase-dependent rotation (14). Consequently, the angular velocities observed with this assay resulted from the intrinsic mechanism of the motors rather than from viscous drag on the optical probe.

Most details concerning the angular velocity during the power stroke are not revealed by measurements of the overall rotation rate because that rate is limited by the duration of the catalytic dwell. These motors can lapse into long pauses that can last as long as 30 s due to formation of an inactive state known as the ADP-Mg form (16). However, under conditions that minimize the occurrence of this inactive state, the average duration of the *Ec* $\alpha_3\beta_3\gamma$ catalytic dwell was determined to be ~8 ms (26). This is consistent with a k_{cat} for ATPase activity of 130 s^{-1} such that each ATP is consumed in 7.7 ms. The measured k_{cat} of 4.5 s^{-1} for the ATPase activity of *MmA*₃B₃DF-complex presented here (Figure 3B) is then comparable to a catalytic dwell of ~220 ms. Since both *Ec* $\alpha_3\beta_3\gamma$ and *MmA*₃B₃DF-complexes rotate in 3 consecutive 120° power strokes equivalent with the consumption of one ATP (Figure 4), the overall rotation rates of these motors are on the order of 43.3 rps ($1/3(0.0077 \text{ s})$) and 1.5 rps ($1/1/3(0.220 \text{ s})$), respectively. The catalytic dwell of the *Gs* $\alpha_3\beta_3\gamma$ has been determined to be ~2 ms, which corresponds to a rotation rate of ~166 rps at saturating ATP (Adachi *et al.* Cell 130, 309-321). In contrast, the duration of the power strokes of the motors presented here is on the order of 0.2-0.4 ms, and thus the power strokes do not contribute substantially to the overall rate of rotation.

Possible factors that limit the angular velocity of the power stroke at a given rotary position include the binding affinity for MgATP, the mechanical efficiency of MgATP-binding induced closure of the catalytic site lever acting on the axle as a cam shaft, and the drag of the axle as it rotates within the hexagonal ring of catalytic subunits. The observed effects of limiting ATP, and of elevated ADP, on the average angular velocity of the *Ec* $\alpha_3\beta_3\gamma$ power stroke were not significantly changed when power stroke data were analyzed by arcsine^{1/2} presented here versus arcsine (8). However, the increased resolution of angular velocity achieved with arcsine^{1/2} analysis during the initial 20° of the power stroke revealed that high ADP also suppressed the angular velocity in this region.

Energy derived from the binding of substrate to the empty catalytic site of *Ec* $\alpha_3\beta_3\gamma$ is known to contribute substantially to the angular velocity of the rotor since MgITP, which has a lower binding affinity than MgATP, results in lower velocities than MgATP (8). The binding of MgATP to the empty catalytic site of F₁-ATPases results in the closure of the lever domain to make contact with the drive shaft. Pu and Karplus (23) modeled variations in torque during an F₁ power stroke as a function of rotational position using a simple functional form to calculate potential energy coupling of the action of the levers on the drive shaft during targeted molecular dynamics (TMD) trajectories. The variations in torque derived from these TMD simulations (23) were subsequently found (8) to fit well with the profile of accelerations and decelerations of the *Ec* $\alpha_3\beta_3\gamma$ power stroke at rotary positions 52°, 88°, 91°, 94°, and 97° in Figure 7. This fit supports the hypothesis that rotation is powered by van der Waals repulsive forces from MgATP-binding dependent movement of the lever acting against the drive shaft as a cam during the final 85° of the power stroke (8).

The angular velocities reported here are similar during the final 50° of the power strokes of the *MmA*₃B₃DF-complex from the A-ATP synthase, the *Gs* $\alpha_3\beta_3\gamma$ -complex, and that of *Ec* $\alpha_3\beta_3\gamma$ (Figure 7A). This suggests that rotation of the A-type is also powered by MgATP-binding dependent movement of the lever acting against the drive shaft as a cam in a manner similar to that of the F-type motors. However, while F-type motors bind MgATP 40° after the catalytic dwell (15,18), the results presented here (**Figure 8**) show that the *MmA*₃B₃DF-complex does not exhibit an ATP-binding dwell at limiting MgATP concentrations. Bacterial V-ATPases of *T. thermophilus* and *E. hirae* also do not

exhibit an additional ATP-binding dwell at limiting ATP concentrations (21,24), suggesting that ATP-binding occurs at the same rotary positions as the catalytic dwell in bacterial V-ATPase A_3B_3DF -complexes (22). Using conditions that controlled the rotary position (30), $Gs\alpha_3\beta_3\gamma$ was found to bind MgATP over a range of rotary positions with increasing affinity as a function of the extent of CCW rotation from the catalytic dwell. Although the A-type motors bind MgATP at the catalytic dwell, the close similarities in the angular velocities as a function of rotary position reported here may be possible if the mechanical efficiency of lever closure has a similar dependence on rotary position to that of the F-type motors.

As shown in Figure 6, the average angular velocity powered by 1 mM MgATP of $Ec\alpha_3\beta_3\gamma$ was 28% higher during the first 70° compared with that of thermophilic $Gs\alpha_3\beta_3\gamma$ and the archaeal MmA_3B_3DF . This suggests that the transfer of substrate binding in $Ec\alpha_3\beta_3\gamma$ into angular velocity is more efficient than in $Gs\alpha_3\beta_3\gamma$ or in MmA_3B_3DF . Differences in substrate binding affinity and in the drag imposed on the axel by the inner surface of the ring of catalytic subunits may explain these effects. The slower angular velocities reported here are consistent with the lower values of torque reported for $Gs\alpha_3\beta_3\gamma$ (31) than for $Ec\alpha_3\beta_3\gamma$ (17,32).

Elevated ADP concentrations have been found to affect ATPase-dependent rotation of $Ec\alpha_3\beta_3\gamma$ in two ways (8). First, ADP can induce an ADP-binding dwell $\sim 40^\circ$ after the catalytic dwell, which has also been observed in $Gs\alpha_3\beta_3\gamma$ (15). Second, high ADP concentrations decrease the angular velocity of the $Ec\alpha_3\beta_3\gamma$ power stroke in a manner not related to dwells (Figure 9C). Martin *et al.* (8) concluded that ADP-binding dwells resulted from the competitive binding of ADP with ATP for the empty catalytic site (β_E), while the decreases in angular velocity not related to dwells were the result of the decreased ability of product ADP to be released from the β_D site. In response to elevated ADP, the MmA_3B_3DF -complex did not exhibit ADP-binding dwells 40° after the catalytic dwell, nor was the angular velocity changed significantly (Figure 9D).

The mechanistic differences between MmA_3B_3DF and $Ec\alpha_3\beta_3\gamma$ at limiting ATP and/or at elevated ADP are summarized in Figure 10. In both complexes ATP hydrolysis and phosphate release occur

during the catalytic dwell. In bacterial F-type ATPases, these steps occur at the β_D and β_E catalytic sites, respectively, and the power stroke is initiated by the release of phosphate. When ATP is limiting, the power stroke of the bacterial F-type ATPases is halted after the axle has rotated 40° CCW from the catalytic dwell by an ATP-binding dwell during which time nucleotide does not occupy the β_E site. An ADP-binding dwell can also occur 40° after the catalytic dwell if ADP outcompetes ATP for binding to the β_E site, which occurs more frequently when ADP is elevated. The power stroke resumes for 80° once ATP has displaced ADP from the β_E site. However, in the presence of elevated ADP, the angular velocity of the power stroke is also suppressed, presumably because dissociation of ADP from the β_D site is inhibited due to mass action. In contrast, the binding of ATP or ADP to the empty catalytic site (A_O) of the *MmA*₃*B*₃*DF* complex occurs during the catalytic dwell, as does ATP hydrolysis, and release of ADP from the catalytic site (A_{CR}) that is putatively equivalent to the β_D site of the F-type ATPase (37). In Figure 10A, experimental evidence supports a compulsory ordered mechanism for bacterial F-ATPases that includes: (a) ATP hydrolysis at β_D ; (b) Pi release at β_E ; (c) ATP binding at β_E ; (d) ADP release from β_D concurrent with, or after ATP binding (29). These four events are illustrated in the same order in Figure 10B, to indicate that they all occur during the catalytic dwell, and not to imply that the order of these events is the same in A-type ATPases as in bacterial F-ATPases.

EXPERIMENTAL PROCEDURES

Materials

Pfu DNA polymerase was purchased from Thermo Scientific (Massachusetts, USA) and Ni-NTA chromatography resin was obtained from Qiagen (Hilden, Germany). Enzymatic digestion was performed using restriction enzymes from New England BioLabs. Chemicals from Bio-Rad (California, USA) were used for SDS-PAGE. All other chemicals of analytical grade were obtained from Biomol (Hamburg, Germany), Merck (Darmstadt, Germany), Sigma-Aldrich (Missouri, USA), or Serva (Heidelberg, Germany).

Genetic engineering of the MmA₃B₃DF-complex.

The *MmA₃B₃DF-M1* protein included the amino acid substitutions C36V, C72V, C181A and C380V in subunit A as well as the substitutions C67S and A71C in subunit B and D, respectively. The fragment containing genes *ahaA*, *ahaB*, *ahaF*, and *ahaD* of *MmA₃B₃DF-M1* were synthesized and provided by *DNA 2.0* in the vector pJ367 (in-house cloning vector). The fragment was further sub-cloned into pGEM-4Z (33). The pGEM-4Z vector was kindly provided by Prof. V. Müller (Johann-Wolfgang Goethe University, Frankfurt, Germany). The mutant protein *MmA₃B₃DF-M2* subsequently designated *MmA₃B₃DF*, contained the single substitution A71C in subunit D, which was generated by replacing the gene encoding subunit D of *MmA₃B₃DF-WT* with the gene encoding subunit D mutation A71C of *MmA₃B₃DF-M1* in pJ367. The replacement of subunit D was possible because of the presence of an endogenous *NcoI* site at the N-terminal of subunit D, which was then used along with *SacI*. All constructs contained a His₈-tag at the N-terminus of subunit A and the respective proteins were produced in *E. coli* DK8 cells. Protein complex production and purification of both mutant complexes were performed as described previously (25).

Purification of the Gs $\alpha_3\beta_3\gamma$ -complex

The *Gs $\alpha_3\beta_3\gamma$* -complex was purified according to Cs *et al.* (34). Briefly, Ni²⁺-NTA affinity chromatography was performed with a linear imidazole gradient of 20-600 mM. Fractions containing the *Gs $\alpha_3\beta_3\gamma$* -complex were pooled and applied onto an anion exchange column (Resource Q, 6 ml, GE-Healthcare) with a buffer containing 50 mM Tris/HCl, pH 7.5, 50 mM NaCl. After increasing the NaCl concentration stepwise the protein eluted at 250 mM NaCl. Protein containing fractions were concentrated using a Centricon YM-100 (100 kDa molecular mass cut off) spin concentrator (Millipore) and stored at -80 °C.

ATP hydrolysis assay

The rates of ATPase activity were measured in a NADH coupled assay. Samples were added at 37 °C to 1 ml of a reaction mix, containing 25 mM Hepes/HCl, pH 7.5, 25 mM KCl, 5 mM MgCl₂, 5 mM KCN, 2 mM phosphoenolpyruvate, 2 mM ATP, 0.4 mM NADH, 30 U L-lactic acid dehydrogenase and 30 U pyruvate kinase. The absorbance at 340 nm was observed with spectrometer for 5 min. The activities were calculated from the initial linear slope.

Labeling of MmA_3B_3DF and $Gs\alpha_3\beta_3\gamma$ -complexes

The cysteine C71 in subunit D of MmA_3B_3DF -M2 and the cysteine C109 in the γ subunit of $Gs\alpha_3\beta_3\gamma$ were biotinylated for the attachment of a neutravidin-coated gold nanorod. Biotinylation was accomplished by 1 min incubation at 4 °C of 12.7 μ M of MmA_3B_3DF -M2 with a 1.5-fold molar excess of biotin-PEAC₅-maleimide (Dojindo, Japan) in 25 mM HEPES/HCl, pH 7.5, 25 mM KCl, 5 mM MgCl₂. In case of $Gs\alpha_3\beta_3\gamma$, freshly prepared protein was incubated for 5 min at 4 °C with a 1.2-fold molar excess of biotin-PEAC₅-maleimide in 250 mM NaCl and 50 mM Tris/HCl, pH 7.5. The reaction for both complexes was stopped by adding 2 mM acetyl-cysteine, and unbound biotin-maleimide was removed by dilution, followed by filtration in a centrifuge (Centricon YM 100). Samples were frozen in liquid N₂ and stored at -80 °C prior to use.

Single molecule rotation assays

Samples were pipetted into flow cells consisting of two coverslips, separated by double-adhesive tape. Prior to use, the bottom coverslip was cleaned for 30 min by sonication in 1% Hellmanex III, followed by rigorous washing with H₂O. Gold nanorods (40x76 nm, Nanopartz, USA) were prepared by mixing 9 μ l of nanorods with 1 μ l Neutravidin (5 mg/ml in H₂O, Molecular Probes, USA) for 1 h before adding 1 μ l BSA (10% solution, Aurion, The Netherlands) for 1 min and 89 μ l of buffer RB (50 mM Tris pH 8.0, 10 mM KCl). Solutions were infused in a flow cell (30 μ l, 5 min incubation) in the following order, with washing steps of 60 μ l buffer RB in between: (1) Ni²⁺-NTA-horseradish peroxidase conjugate (Qiagen, Germany) in deionized H₂O; (2) 1 nM biotinylated protein in buffer RB; (3) gold nanorods in buffer RB; (4) Nucleotide solution in buffer RB.

The gold nanorod attached to the protein served as a visible probe of rotation. Samples were illuminated by a mercury lamp through a dark-field condenser in a confocal microscope (Axiovert, Zeiss, Germany). The intensity of red light scattered from the nanorod was observed through a band pass filter and a polarizer, and detected by an avalanche photo diode (APD) as described previously (27). Data were collected for 10 s and 30 s at 1 kHz and 200 kHz, respectively, and were analyzed using custom software (Matlab, MathWorks) to obtain intensities and velocities at each angular position of the rotating protein-nanorod complex (8). From the light intensity fluctuations recorded by

the APD, power strokes were identified by intensity changes from a minimum taken from the lowest 5 percentile of peaks to a maximum taken from the highest 5 percentile of peak, for which a linear least square fit gave an R-value of 0.9. This aligned the power strokes and minimized the phase shift when averaging the data from many power strokes. This also set the same minimum and maximum intensity values, $I(t)$, for any one molecule so the normalized value satisfied $0 \leq I(t) \leq 1$, which was required to take note of the relation between the rotational position, $\theta(t)$, and the intensity $I(t)$. We let t_0 be such that $I(t_0) = 0$ is the first time at which the intensity is zero. We also let t_{max} be the first time at which $I(t_0) = I_{max}$, (i.e. I is increasing for $t_0 < t < t_{max}$, with $0 < I(t) < 1$). Since the scattered light intensity varies in a sinusoidal manner dependent on the rotational position of the nanorod relative to the direction of polarization, the rotational position during the power stroke was determined from

$$\theta(t) = \arcsin^{1/2} I(t) = \arcsin^{1/2} (I(t)) + \theta(t_0) \quad (\text{Eq. 1})$$

for $t_0 < t < t_{max}$, which is valid for the first 90° of the 120° power stroke ($-\pi/2 < \theta < \pi/2$). To obtain the rotational position from the scattered light intensity for the final 30° of the power stroke, we used

$$\theta(t) = \pi - \arcsin^{1/2} (I(t)) + \theta(t_0) \quad (\text{Eq. 2})$$

for $t > t_{max}$, which is valid for $\pi/2 < \theta < \pi$. The rotational position versus time for each power stroke calculated from Eqs. 1 and 2 was used to determine the average angular velocity, and the occurrence of dwells as a function of rotational position. Determination of rotational position using the arcsine function followed the same procedure as described in Martin *et al.* (8).

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: HS, JM, DS and GB performed the experiments, HS and JM analyzed the data, JM wrote the algorithm. GG and WF conceived the idea for the project, and HS, GG and WF wrote the paper.

FOOTNOTES

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FIGURE LEGENDS

FIGURE 1. Comparison of the related lever- and foot domains of the *MmA*₃*B*₃*DF*- and F-type $\alpha_3\beta_3\gamma$ -complexes. **(A)** Composite structure of subunits A, B, D and F in the *MmA*₃*B*₃*DF*-complex of the A-ATP synthase. The catalytic domain (*gray ribbon*) and the lever domain (*orange*) of subunit A (PDB ID 3I4L (35)) in the *A*₃*B*₃ hexamer that surround the central stalk subunits formed by subunit-F (*magenta*, PDB ID 2OV6 (36)) and subunit-D (*yellow*, PDB ID 3AON (37)). **(B)** Structural components of the $\alpha_3\beta_3\gamma$ complex of bovine mitochondrial F-ATP synthase (PDB ID 1E79) (38) showing the catalytic domain (*gray ribbon*) and lever domain (*purple*) of β subunits that surround the coiled-coil (*green*) and foot (*blue*) domains of the γ -subunit. Subunits B and α are omitted for clarity.

FIGURE 2. Rotary mechanism of bacterial F ATPases at saturating ATP concentrations. The ($\alpha_3\beta_3$)-ring (viewed from the membrane side) is designated as a ring of α -subunit (*gray*) and β -subunit (*white*) hexagons that surround the γ -subunit composed of the coiled-coil (*green*), and foot (*red*) domains. During the catalytic dwell ATP hydrolysis occurs at β_D (a), followed by Pi release at β_E (b) that initiates the power stroke (c). At saturating ATP concentrations, the power stroke is continuous for 120° (c) during which time ATP binding to β_E and ADP release from β_D occur. Upon formation of the next catalytic dwell (d), β_T changed conformation to β_D to induce ATP hydrolysis.

FIGURE 3. Purification and ATPase activity of *MmA*₃*B*₃*DF* complexes. **(A)** 17% PAGE-SDS gel (39) of the purified recombinant protein complexes with the respective molecular weight marker. **(B)** Specific ATPase activities of protein complexes as determined from the initial slope of the decrease in NADH absorbance in the coupled ATPase assay.

FIGURE 4. Single-molecule assay of rotating protein-gold nanorod complexes. **(A)** Schematic model of the microscope setup with a protein-gold nanorod complex. The *MmA*₃*B*₃*DF* complex (subunits A, B, D, and F in orange, green, yellow, and purple, respectively) is attached via its His₈-tags in subunit A to a cover slip, while a neutravidin-coated nanorod is attached to the biotinylated cysteine (*red*) in

subunit D. Gold-nanorods were illuminated by a dark-field condenser. Red scattered polarized light was recorded by an APD after passing through a polarizer and a band pass filter to block shorter wavelengths of light. **(B)** Consecutive histograms of light intensity scattered from a nanorod attached to a rotating *MmA₃B₃DF* complex collected at 1 kHz as a function of the polarization angle during ATPase-powered rotation. **(C)** 9% PAGE-SDS gel of purified recombinant *Gsα₃β₃γ* and molecular weight markers. **(D)** Consecutive histograms of light intensity scattered from a nanorod attached to a rotating *Gsα₃β₃γ* complex collected at 1 kHz as a function of the polarization angle during ATPase-powered rotation. The approximate courses of the three sinusoidal curves that resulted from the three catalytic dwells are indicated in grey.

FIGURE 5. Power stroke analysis. **(A)** Light intensities as a function of time collected at 200 kHz during a single power stroke event scattered from a rotating nanorod attached to a single *MmA₃B₃DF* complex in the presence of 1 mM ATP. Starting from a catalytic dwell where the nanorod was aligned with the polarizer such that the scattered light intensity was at a minimum, the intensity increased through a maximum during the power stroke (red) until it reached the subsequent catalytic dwell. **(B)** The rotational position of the power stroke from **(A)** as a function of time calculated from the arcsine^{1/2} function.

FIGURE 6. Comparison of arcsine versus arcsine^{1/2} functions to calculate the rotational position of a gold nanorod from the scattered light intensity as a function of the orientation of the polarizer. **(A)** The average light intensity (red) scattered from each of four nanorods as a function of the rotational position of the polarizer for 120° from an intensity minimum, collected at 1 kHz in successive histograms as per Fig. 4. Each nanorod was attached to a molecule of *Ecα₃β₃γ* that was not rotating. The data were fitted using the arcsine (black) versus the arcsine^{1/2} (blue) functions assuming a perfect fit at the first data point, which was the intensity minimum. **(B)** Comparison of the total error of the fit of the data for each nanorod from **(A)** using arcsine (black) versus arcsine^{1/2} (blue) functions. **(C)** The fits of the experimental data (red) from **(A)** using arcsine (black) versus arcsine^{1/2} (blue) functions

assuming a perfect fit at 3° after the first data point. **(D)** Comparison of the total error of the fit of the data for each nanorod from **(B)** using the arcsine (black) versus the arcsine^{1/2} (blue) functions.

Figure 7. Average angular velocity profiles of the 120° power stroke at 1 mM MgATP. **(A)** Average angular velocities of the ATPase-dependent power strokes of *MmA₃B₃DF* (blue), *Gs $\alpha_3\beta_3\gamma$* (orange), and *Ec $\alpha_3\beta_3\gamma$* (black). **(B)** Comparison of the angular velocity profile of *Ec $\alpha_3\beta_3\gamma$* calculated from the arcsine (red) or the arcsine^{1/2} (black) functions. Data were collected and the rotational position determined as described in Figure 2. Angular velocities were determined from the moving average of the slope of 3 consecutive rotational positions from individual power strokes that were averaged and binned for every 3° .

FIGURE 8. The effects of ATP concentrations that limit the rate of ATPase activity on the average angular velocity profiles. **(A)** Dwell abundance as a function of rotational position during the power stroke of *Ec $\alpha_3\beta_3\gamma$* at 1 mM MgATP (black) versus 0.3 mM MgATP (red). **(B)** Dwell abundance as a function of rotational position during the power stroke of the *MmA₃B₃DF* complex at 1 mM MgATP (blue) versus 0.1 mM MgATP (red). **(C)** Average angular velocity profile of the *Ec $\alpha_3\beta_3\gamma$* power stroke at 1 mM MgATP (black) versus 0.3 mM MgATP (red). **(D)** Average angular velocity profile of the *MmA₃B₃DF* power stroke at 1 mM MgATP (blue) versus 0.1 mM MgATP (red).

FIGURE 9. The effects of elevated ADP concentrations on the average angular velocity profiles. **(A)** Dwell abundance as a function of rotational position during the power stroke of *Ec $\alpha_3\beta_3\gamma$* at 1 mM MgATP in the absence (black) versus the presence of 4 mM MgADP (red). **(B)** Dwell abundance as a function of rotational position during the power stroke of the *MmA₃B₃DF* complex at 1 mM MgATP in the absence (blue) versus the presence of 50 μ M (red), and 250 μ M (orange) MgADP. **(C)** Average angular velocity of the power stroke as a function of rotational position of *EcF₁* at 1 mM ATP in the absence (black) versus the presence (red) of 4 mM MgADP. **(D)** Average angular velocity of the

power stroke as a function of rotational position of the *MmA*₃*B*₃*DF* complex at 1 mM MgATP (blue) in the absence (blue) versus the presence of 50 μM (red), and 250 μM (orange) MgADP.

Figure 10. Mechanistic differences between *MmA*₃*B*₃*DF* and the *Ec*α₃β₃γ at limiting ATP and/or at elevated ADP concentrations. **(A)** The (α₃β)₃-ring of the *Ec*α₃β₃γ complex is designated as a ring of α-subunit (grey) and β-subunit (white) hexagons surrounding the γ-subunit composed of the coiled-coil (green), and the foot (red) domains. During the catalytic dwell ATP hydrolysis occurs at β_{DP} (a), followed by Pi release at β_E (b) that initiates the power stroke. At limiting MgATP concentrations (c), an ATP-binding dwell interrupts the power stroke at 40° when ATP has not bound to β_E. At elevated ADP concentrations (d), an ADP-binding dwell interrupts the power stroke at 40° when ADP out-competes ATP for binding to β_E. The power stroke resumes for 80° upon binding of ATP to β_E (e). Elevated ADP concentrations limit the power stroke angular velocity (yellow) by delaying release of ADP from β_{DP} (f). Upon formation of the next catalytic dwell, β_{TP} changed conformation to β_{DP} to induce ATP hydrolysis (g) that will be continued by the next 120° power stroke (not shown). **(B)** The (AB)₃-ring of the *MmA*₃*B*₃*DF* complex is designated as a ring of A-subunit (white) and B-subunit (grey) hexagons surrounding the central stalk composed of the D-subunit (green), and F-subunit (red). During the catalytic dwell ATP hydrolysis (a), phosphate release (b), ADP release (c) and ATP binding (d) occur in an, as yet, unspecified order. The 120° power strokes (e) are not interrupted by ATP-binding or ADP-binding dwells. Upon formation of the next catalytic dwell, A_C changed conformation to A_{CR} to induce ATP hydrolysis (f) that will be continued by the next 120° power stroke (not shown). Sites β_E, β_{DP} and β_{TP} in *Ec*α₃β₃γ **(A)** correspond to sites A_O, A_{CR} and A_C in *MmA*₃*B*₃*DF* **(B)** (37).

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