Real time imaging of rotating molecular machines

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ABSTRACT **Observation of true rotation has been relatively rare in living systems, but there may be many molecular machines that rotate. Molecular rotations accompanying function can be imaged in real time under an optical microscope by attaching to the protein machine either a small tag such as a single fluorophore or a tag that is huge compared with the size of the protein. As an example of the former approach, axial rotation of an actin filament sliding over myosin has been measured quantitatively by attaching a fluorophore rigidly to the filament and imaging the orientation of the fluorophore continuously by polarization microscopy. As a huge tag in the latter approach, an actin filament turned out to be quite useful. Using this tag, the enzyme F_1-ATPase has been shown to be a rotary stepper motor made of a single molecule. Further, the efficiency of this ATP-fueled motor has been shown to reach almost 100%. The two examples above demonstrate that one can now image conformational changes, which necessarily involve reorientation, in a single protein molecule during function. Single-molecule physiology is no longer a dream.—Kinosita, Jr., K. Real time imaging of rotating molecular machines.** ***FASEB J.*** **13 (Suppl.), S201–S208 (1999)**

ROTATION OVER INFINITE angles of one part against another requires that the two parts slide against each other and hence that the two be disconnected from each other. Thus, rotation is inconceivable at the level of an individual, except for apparent rotations such as the swing of an arm, which is actually a combination of two torsional motions. Connectivity, however, is ill defined at the level of molecules, because noncovalent bonds between molecules are continually broken and formed at body temperature. Lipid bilayer is a typical example in which molecules freely diffuse against one another yet keeping the overall integrity of the membrane. As a result, one part of a cell can rotate indefinitely against the other without breaking the cell membrane, as has been well demonstrated for a protozoan (1) and sperm (2). Such rotations at the cellular level, however, appear to be rare exceptions, because in most cases the cell membrane is backed up by cytoskeletal network that does not readily disintegrate into pieces.

Protein-protein interactions are stereospecific, involving many noncovalent bonds that act cooperatively. Relatively strong associations between proteins, however, are often modified by specific ligands such as a nucleotide, leading to rapid alternation of binding and unbinding. If one of the partners is an array of protein molecules, sliding may ensue, as exemplified by molecular motors. Sliding is an essential ingredient of rotation. On the other hand, protein arrays are often helical, including disks and rings, because Nature has created only limited kinds of proteins and because repeated use of identical molecules in identical manners results, in general, in a helical symmetry. Sliding along a helical array produces rotation. Thus, rotation may be quite common at the level of protein molecules. However, there have been few protein machines that have been proved to rotate, possibly because of technical difficulty in observing rotation. Here we demonstrate that molecular rotations can be visualized under an optical microscope by the use of either a small or huge tag.

HELICAL ROTATION OF A SLIDING ACTIN FILAMENT

An actin filament is made of a two-start, right-handed helix with a pitch of ~72 nm (ref. 3; **Fig. 1**a). When the helical filament is moved by myosin, a linear motor, one may expect that the filament rotates around its axis. Indeed, Nishizaka et al. (4) have shown that a sliding actin filament does rotate as a right-handed screw as anticipated from the sense of the actin helix. They, however, were unable to quantitate the axial rotation because of the lack in spatial resolution in their microscopic images. We therefore attempted to visualize the axial rotation by the use of a small tag, a single fluorophore (5).

As shown in Fig. 1*b*, the orientation of a fluorophore can be assessed by decomposing the fluorescence from the fluorophore into vertically (V) and

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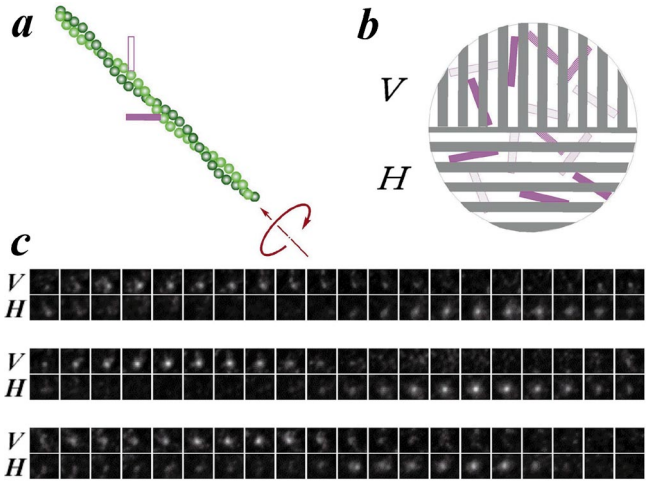

Figure 1. Visualization of the axial rotation of a sliding actin filament. *a*) Schematic diagram of the experiment. Violet bars represent the orientation of the fluorophore attached to the actin filament. *b*) Polarized dual-view observation of the orientations of fluorophores. *C*) Sequential images, at 33-ms intervals, of a single tetramethylrhodamine fluorophore bound to an actin filament sliding from bottom right toward top left. Adapted from ref. 5 (copyright, 1997, National Academy of Sciences, USA).

horizontally (H) polarized components and imaging the two components side by side on a video camera (6). When the fluorophore is vertically oriented, it shows up in the vertical (V) image, and when it is in the horizontal orientation, it appears in the horizontal (H) image. In this way we can determine the fluorophore orientation continuously in real time until the fluorophore is eventually photobleached.

The fluorophore tetramethylrhodamine covalently bound to Cys374 of actin turned out to lie at ~45° from the filament axis without wobbling much on actin (5) (Fig. 1*a*). If we let an actin filament slide at 45° in the image plane, and if the filament rotates as it slides over myosin molecules distributed on a glass surface, then the orientation of the fluorophore in the image plane should alternate between V and H (Fig. 1*a*). Indeed, we observed alternate appearance in the V and H images of the fluorescence from a single fluorophore on an actin filament sliding from bottom right toward top left (Fig. 1*c*). The regular alternation indicates regular rotation at a rate of about two turns per second.

Sliding of a helical array of actin against myosin thus produced rotation as expected. The pitch of rotation, however, did not match the pitch of the actin helix. Analysis of 17 actin filaments showed an average speed of linear sliding of ~2 μm/s, whereas the average rotational rate was ~2 turns/s (5). Thus, the actin filaments make a turn every sliding distance of 1 μm. This rotational pitch of 1 μm is ~15 times as large as the helical pitch of 72 nm. If myosin molecules had tenaciously followed the helical track on an actin filament, the filament should have rotated much faster, ~15 turns/μm or ~30 turns/s. The observed inefficiency in rotation implies that a myosin molecule interacts with an actin filament intermittently, skipping at least ~36 nm (half pitch of the actin helix) between successive bindings. On the surface of an actin filament, a myosin molecule with its two 'feet' (the two actin-binding domains of myosin usually referred to as 'heads') 'runs' rather than 'walks.' If myosin were to walk without detaching its two feet simultaneously, as in the case of kinesin, it would stay on one of the two helical strands of an actin filament, because simultaneous landing of the two feet on the opposing two strands is sterically improbable.

Note that a running myosin molecule does not jump to the next landing site if the myosin is alone. A myosin molecule can move to a distant site on actin because other myosin molecules pull the actin filament while the former is detached. If a single myosin molecule not attached to glass interacts with actin, the myosin will only hop into the solution and will then undergo random diffusion.

Our result that the axial rotation of an actin filament was rather slow also implies that the force generated at the myosin-actin interface is primarily along the filament axis, because an oblique force would rotate the filament efficiently. One might argue that the rotational component of the oblique force may be obstructed by the other myosin molecules that are simultaneously bound to the same filament, but the obstruction also operates on the linear sliding. The driving force, either linear or rotational, is the net sum of the forces by all myosin molecules bound, of which some exert positive and the others negative forces.

CIRCULARLY ROTATING MOLECULAR MACHINES

Helical structures are abundant in cells. Actin and DNA are typical examples, and microtubules are also helical although their normal pitch is long. Thus, helical rotation may well be found in many places in a cell. Protein machines working along DNA are most likely to rotate, although direct evidence has not been obtained.

Rings and disks are relatively scarce. Until recently, the almost sole example of circularly rotating molecular machines was the bacterial flagellar motor (7). This motor is assembled at the root of a flagellum and consists of several rings containing a total of $\sim 10^2$ protein molecules (8, 9). Flow of protons through the motor causes rotation of the flagellum, which propels the bacterium. The rotation was first revealed in a tethered cell (10) where the cell body

served as a huge indicator of the motor rotation; when the flagella were attached to a glass surface, the cell body was seen to rotate under an optical microscope. Using a similar technique, we have proved the existence of another circularly rotating machine, the F_1-ATPase (11).

F_1-ATPase: A ROTARY MOTOR MADE OF A SINGLE MOLECULE

The fit among subunits of a protein molecule is generally considered to be quite snug. Normally they do not separate from one another. Thus, when P. Boyer proposed almost 20 years ago that a single molecule of the enzyme ATP synthase may be a rotary motor in which one or more subunits rotate against the others (12), few people would believe it. Now we know he was right.

The ATP synthase synthesizes ATP from ADP and phosphate using the flow of protons as the energy source. This enzyme is ubiquitous from animals to plants and bacteria. In animals, ATP synthase resides in the inner mitochondrial membrane. Protons are ejected from mitochondria by the action of a chain of protein machines, and the protons flow back into mitochondria through the ATP synthase, where ATP is produced (13). As shown in **Fig. 2**, the ATP synthase consists of a membrane-embedded portion called F_0 and a protruding portion F_1. Protons flow through the F_0 portion, and the flow somehow leads to the synthesis of ATP in the F_1 portion. The ATP synthase is a completely reversible molecular machine in that the hydrolysis of ATP in F_1 drives protons in the reverse direction through F_0.

The mystery is how the proton translocation in F_0 is coupled to the chemical synthesis/hydrolysis of ATP in F_1. Boyer's proposal (12, 14, 15) was that the coupling is mechanical. F_0 and F_1 are each rotary motors, one driven by the proton flow and the other driven by ATP hydrolysis, and the rotating shaft is common to the two. When the energy driving the proton flow (the difference in the electrochemical potential of protons between the two sides of the membrane) is larger than the free energy obtained from ATP hydrolysis, F_0 drives the shaft in its genuine direction; the ATP-driven motor, F_1, is forced to rotate in its reverse direction, resulting in ATP synthesis. If the energy for ATP hydrolysis is larger, the shaft rotates in the opposite direction, and the reverse rotation of the F_0 motor drives protons back against the electrochemical gradient. (In cells, this back reaction tends to be inhibited by a built-in mechanism.)

One can isolate the F_1 portion as a soluble protein, and then it only catalyzes ATP hydrolysis, not synthesis. Hence, the isolated F_1 is called F_1-ATPase. The

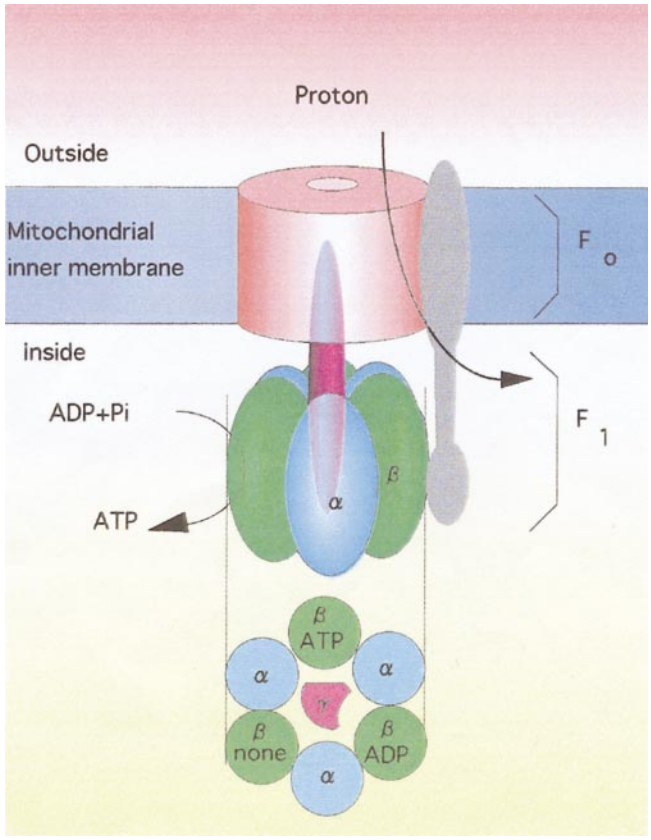

Figure 2. Schematic diagram of the ATP synthase. The gray part is the putative stator for the F_0 rotor. Adapted from ref. 21 (copyright, 1998, Cell Press).

subunit composition of F_1-ATPase is $\alpha_3\beta_3\gamma\delta\varepsilon$, and the ATPase activity resides in the three βs (one catalytic site per β, close to an α-β interface). It was this subunit composition, together with the fact that the three βs participate equally in steady-state ATP synthesis/hydrolysis, that constituted a basis for the Boyer's proposal. The single-copy subunits—γ, δ, and ε—cannot each have a three-fold symmetry, yet the three βs are equivalent in long-term catalysis. In particular, γ is essential for catalysis and thus is not a peripheral accessory. For the asymmetric γ to interact with the three βs impartially, γ has to rotate, visiting the three one by one.

When a crystal structure of F_1-ATPase was solved by J. Walker et al. (16), Boyer's proposal became realistic. As shown schematically in Fig. 2, $\alpha_3\beta_3$ hexamer forms a cylinder, and γ occupies the central hole (part of γ, and δ and ε were not resolved in the atomic structure). The interface between the central γ and the hexamer wall is mostly hydrophobic, suggesting that the wall constitutes an oily sleeve for the putative rotor γ (16). F_1 alone would thus work as a motor when fueled with ATP.

The structure immediately suggested ingenious experiments that would support the rotational catalysis model. In one series (17, 18), a particular residue on γ was crosslinked to one of the three βs.

Then the link was cut and the enzyme was allowed to catalyze ATP hydrolysis (F_1 preparation) (17) or synthesis (whole ATP synthase) (18). After that, the residue was again crosslinked to a β. The second crosslinking was found to be made to any of the three βs, indicating that the residue on γ faces all three βs equally during catalysis. In experiments where catalysis was not allowed between the two crosslinking treatments, the second target was the same as the first one. In another set of experiments (19, 20), a fluorescent dye was attached to γ. Time-resolved polarization measurement showed that the fluorophore changes its orientation over many degrees when the enzyme undergoes ATP hydrolysis reaction. In the most recent experiment (20), the fluorophore appeared to adopt three distinct orientations.

These experiments indicate that γ can rotate within the $\alpha_3\beta_3$ cylinder to the extent that the three βs are impartially visited. This, however, does not necessarily imply that γ continues to rotate in one direction without fluctuating back and forth. Also, the symmetry consideration above that led to the Boyer's proposal does not necessarily point to unidirectional rotation; to-and-fro motions are equally acceptable. Observation of a single F_1-ATPase molecule established that the rotation is mostly unidirectional with only occasional (but significant) backward movement (11, 21, 22).

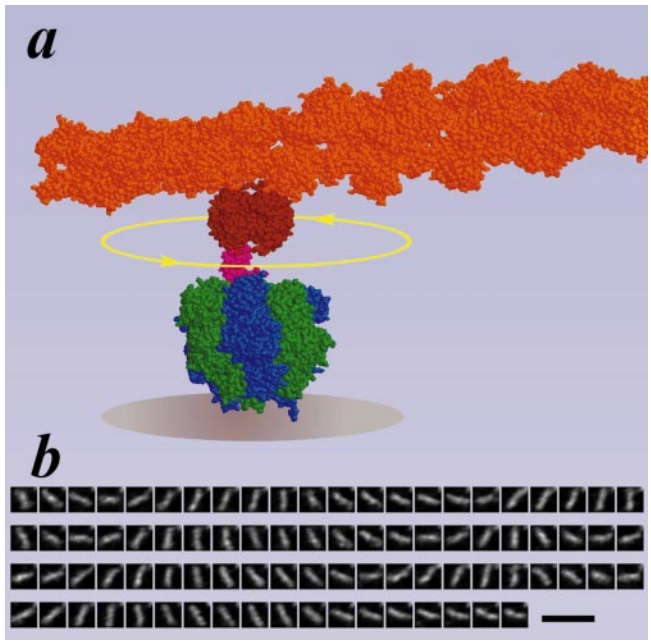

Figure 3. *a*) The experimental system for the observation of the rotation of F_1-ATPase. The space-filling model of F_1 is from ref. 16 (blue, α; green, β; red, γ), actin filament (orange) from ref. 3, and streptavidin (brown) from ref. 23. Streptavidin has four strong binding sites for biotin, and the actin filament is heavily decorated with biotin. There is, however, only one biotin on γ. Presumably, free rotation between streptavidin and γ is prevented by steric hindrance. The diameter of the gray disk, representing the surface to which the three β subunits are bound through histidine tags, is ~22 nm. *B*) Sequential images, at 33-ms intervals, of a rotating actin filament. The average rate of rotation was 1.3 turns/s. Bar = 5 μm. Adapted from ref. 11 (copyright, 1997, Macmillan Magazines).

DIRECT OBSERVATION OF THE ROTATION IN F_1-ATPase

To observe rotation, we constructed a subcomplex of F_1, $\alpha_3\beta_3\gamma$, from a thermophilic bacterium (11). This subcomplex, lacking δ and ε, is sufficient for the ATPase activity and, as shown below, is also sufficient for rotation. Hereafter we refer to this complex simply as F_1.

The putative stator part of F_1, the $\alpha_3\beta_3$ cylinder, was fixed to a glass surface (11) or, more recently, to the surface of a plastic bead (22) such that the binding site for F_0 was away from the surface (**Fig. 3**a). To the putative rotor γ, a fluorescent actin filament was attached as a huge marker through streptavidin-biotin links. A desired concentration of ATP was added, and the sample was examined under a fluorescence microscope. Then we saw fluorescent filaments rotating in one direction (Fig. 3*b*) invariably counterclockwise when viewed from above in Fig. 3*a*. There were many more filaments that did not rotate, presumably because they were bound by more than one F_1 molecules or were obstructed by the surface. But those filaments that rotated continued to do so over many revolutions, often well over hundreds of turns (11). The counterclockwise rotation was powered by ATP; in its absence, continuous rotation was not observed.

The filament in Fig. 3*b* rotated around its center, where a tiny F_1 presumably held and drove the filament. This motion cannot be a pseudo rotation without sliding (as in the case of the swinging of an arm). When one holds the middle of a rod much longer than one's height, one cannot rotate it without releasing the grip occasionally. Thus, a single molecule of F_1 is indeed a molecular motor in which the central γ subunit rotationally slides against the stator hexamer $\alpha_3\beta_3$ over infinite angles. With the size of the stator cylinder of ~10 nm and the diameter of the rotor of ~2 nm, F_1 is the world's smallest rotary motor ever known.

The counterclockwise rotation is consistent with the crystal structure of F_1 (16). In the crystal, the three β subunits carried an analog of ATP, ADP, and none in the clockwise order (Fig. 2). If this structure represented a kinetic intermediate in the hydrolysis reaction, the ATP in the first β would, in the next step, be converted to ADP, the ADP in the second β would be released, and the third β would bind ATP from solution. Then the surface of γ that favors the

empty β (convex in Fig. 2, bottom), for example, would rotate toward the second β (i.e., in the counterclockwise direction). It is likely that the crystal structure, obtained in the presence of MgADP in the medium, represents the so-called MgADP-inhibited form of the enzyme (16). This is a kind of product inhibition that cells probably exploit to prevent futile consumption of ATP. The clockwise order of bound nucleotides consistent with the counterclockwise rotation, then, suggests that the inhibited form arises from a normal intermediate. ADP to be released is somehow trapped in the binding site possibly by premature release of phosphate, which leads to tighter binding of ADP.

The observation of individual molecules under a microscope has demonstrated that F_1 is a rotary motor that rotates counterclockwise when it hydrolyzes ATP. We note again, however, that many of the filaments we observed did not rotate, or only fluctuated, and that there were many more F_1 molecules to which an actin filament failed to bind. That is, the conclusions we draw from these experiments apply to those molecules that we somewhat arbitrarily selected to analyze. What we show here is the 'best' performance of F_1 on our artificial surface. Such selection is a weak point of most single-molecule studies but is also a strong point in that one can focus on the best part, which is often buried in experiments that report only the average behavior. It is quite possible, for example, that most of the molecules being measured in a conventional experiment, either *in vitro* or *in vivo*, are in fact dormant without being noticed to be so. Single-molecule observation will detect such heterogeneity and will allow the selective characterization of active ones.

The selection process might cast some doubt on the objectivity of the conclusions. For example, an actin filament bound to a single myosin molecule on a glass surface rotates plus or minus several turns just by diffusion (24), presumably because myosin is a very flexible molecule. If one selects from the video of this fluctuating actin a particular short sequence, one could show that the filament makes a complete, apparently regular, turn, which would be indistinguishable from the rotation driven by F_1-ATPase. Conversely, an actin filament presumably bound to F_1 often exhibits random rotation, and there are occasions where it makes a turn or two, over time, of clockwise rotation. We are tempted to ascribe these irregular motions to thermal diffusion. In contrast, when we saw by eye the long-lasting counterclockwise rotation of an actin filament, we almost immediately believed that it must represent the authentic motor motion, because the motion was too consistent to be of thermal origin. This may not be a perfectly scientific judgement, but those who watch the video may find seeing to be believing.

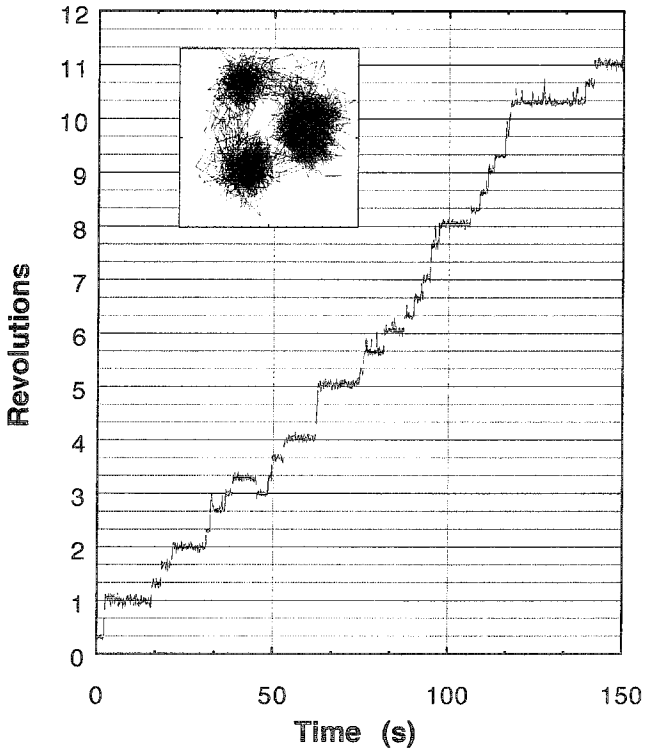

Figure 4. Stepping rotation of F_1 at 20 nM ATP. Inset shows the trace of the centroid of the actin filament. Adapted from ref. 22 (copyright, 1998, Cell Press).

F_1-ATPase IS A STEPPER MOTOR WITH A NEARLY 100% EFFICIENCY

Now we report more on the properties of 'well-behaving' F_1. First, it is a 120°-stepper motor, as may be expected from its structure. Stepping is not resolved in Fig. 3*b* obtained at a high ATP concentration (2 mM). At nanomolar concentrations of ATP, however, the actin rotation became clearly stepwise, as shown in **Fig. 4** (22). Between steps, the F_1 waited for the next ATP molecule to come. The structure of F_1 in the waiting phase appears to be stable for many seconds, at least as long as the orientation of γ is concerned. Metastable orientations of γ other than those separated by 120° were not noticeable at the time resolution of 5 ms.

The intervals between steps were irregular, as expected for stochastic binding of ATP. The histogram of the intervals was exponential with short intervals being more abundant than long ones. This indicates that each step is driven by one ATP molecule (22). If two or more ATP molecules were required per step, short intervals would be scarce because simultaneous binding of two ATP molecules should be a rare event.

Each step is made in a time of the order of 0.1 s. If F_1 is scaled to the size of a person, the person standing at the bottom of a swimming pool would be rotating a rod some 300 m long at the speed of

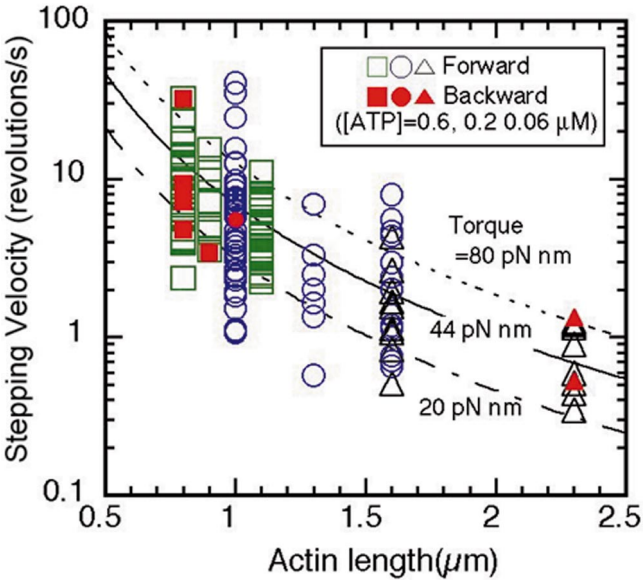

Figure 5. Stepping velocities in individual steps, in revolutions per second, at indicated [ATP]. Smooth lines show the velocities (v) expected when F_1 produces a constant torque (N) irrespective of the load: $v = 2\pi N/\xi$, where $\xi = (4\pi/3)\eta L^3/[\ln(L/2r)-0.447]$ is the rotational frictional drag coefficient, L and r are the length and radius of the actin filament, and η ($= 10^{-3}$ N·s·m^{-2}) is the viscosity of water. Red symbols represent backward steps. Adapted from ref. 22 (copyright, 1998, Cell Press).

120° per 0.1 s! The hydrodynamic friction against rotation is enormous. F_1 is really a powerful motor. Naturally, though, the stepping velocity is slower for a longer filament that is subject to a greater friction (**Fig. 5**). The stepping velocities measured in individual steps are highly variable, but, on the whole, they are distributed around the solid line in Fig. 5. The line shows the velocity expected when F_1 produces a constant torque of 44 pN·nm irrespective of the frictional load (actin length). It appears that F_1 is designed to be a constant-torque machine. If the torque of 44 pN·nm is produced at the interface between β and γ, at the radius of 1 nm, the sliding force between β and γ amounts to 44 pN, far greater than the force produced by various linear motors (21).

The torque of 44 pN·nm times $2\pi/3$ radians (120°), ~90 pN·nm, is the work done in a step against the viscous load. Because the torque is constant, the work per step is also constant. *In situ*, probably, the F_1 motor linked to F_0 can do this much of work against F_0 to pump protons. In cells, the free energy for ATP hydrolysis is ~80 pN·nm per one molecule of ATP. Thus, F_1 is expected to work at ~100% efficiency.

We also measured the torque and work at high ATP concentrations where steps were no longer resolved (22). **Figure 6** summarizes the average velocities of actin filaments of different lengths (abscissa) at various ATP concentrations (distinguished by color). The smooth lines in the figure are calculated on the assumptions that *1*) F_1 produces a constant torque of 40 pN·nm irrespective of the load or ATP concentration, *2*) three ATP molecules are consumed per turn, and *3*) the time per one third of revolution is simply the sum of the ATP cycle time at no load (measured without actin) and the time needed to rotate the actin filament by 120° under the torque of 40 pN·nm. The fairly good agreement between the data and calculated lines indicate again that the F_1 motor produces a constant torque of ~40 pN·nm or a constant work per step of ~80 pN·nm. In most of these experiments and those in Fig. 5, the free energy of ATP hydrolysis was not well defined because the concentrations of ADP and phosphate were not controlled. Figure 6, however, contains two data sets where the free energy per one molecule of ATP was set at 110 pN·nm (+ in dark red) and 90 pN·nm (+ in red). These data also agree with the red line, indicating that F_1 under these conditions indeed worked at a near 100% efficiency.

In summary, the F_1 motor is a 120° stepper motor that produces a constant torque of ~40 pN·nm, or a constant work per step of ~80 pN·nm, over a broad range of load and speed. It consumes one ATP molecule per step, and it can operate even when the

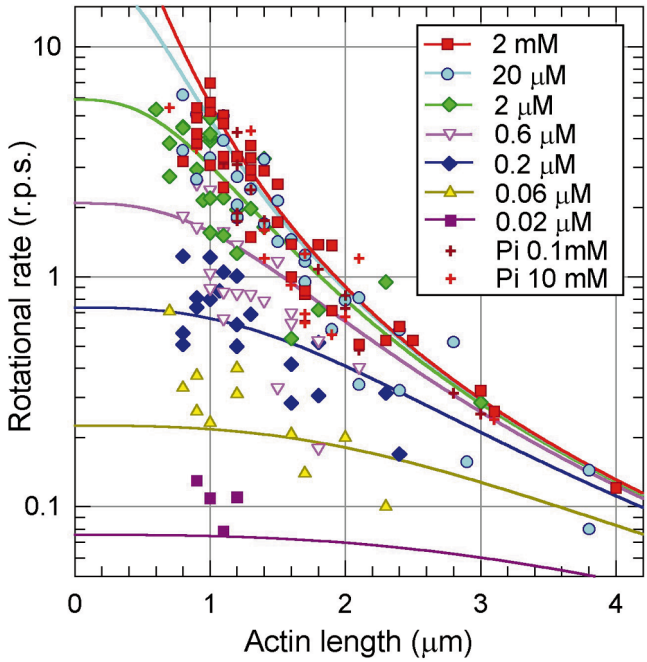

Figure 6. Load (actin length) and ATP dependence of the average velocity of rotation. ATP concentrations are shown in the box. ADP and phosphate concentrations were not controlled except for the + symbols for which [ATP] = 2 mM and [ADP] = 10 μM. Lines are calculated as $1/(3\tau_{120°})$, where $\tau_{120°} = \tau_{ATP} + \tau_{step}$ is the time per 120°step, τ_{ATP} is the ATP cycle time at no load, and $\tau_{step} = (2\pi/3)\xi/N$, where N=40 pN·nm and ξ is the drag coefficient (see legend to Fig. 5). τ_{ATP} was estimated from the rate of ATP hydrolysis in solution without bound actin. Adapted from ref. 22 (copyright, 1998, Cell Press).

free energy of ATP hydrolysis is almost equal to the work to be done.

Another interesting feature is that the motor occasionally makes back steps, as seen in Fig. 4. The back steps are as fast as forward ones (red symbols in Fig. 5), suggesting that these back steps also consume an ATP molecule.

MECHANISM OF F_1 MOTOR

The rotation of F_1 is clearly stepwise at nanomolar ATP concentrations. In this concentration range, F_1 in the waiting phase between steps is considered to carry only one nucleotide among the three βs, in an equilibrium between ATP and ADP plus phosphate. When a second ATP binds to an empty β, it induces rapid release of ADP and phosphate from the first site, resulting in the net hydrolysis of one ATP molecule. The central γ is supposed to rotate during this cooperative catalysis or during the seesaw action between the two binding sites (14, 15).

To ensure the counterclockwise rotation, the second ATP has to bind to the correct β, the one in the counterclockwise direction from the first β. Presumably, it is the central, asymmetric γ that controls the affinity of peripheral βs for nucleotides. The affinity of the correct β for ATP is made higher, but, probably, the affinity of the other empty β is also appreciable and binding of ATP to this wrong β causes the backward step.

Conversely, the orientation of γ is dictated by the peripheral βs. The βs change their conformations depending on the bound nucleotide, and the conformations of the three βs together determine the most stable orientation of γ. In the F_1 in the waiting phase, only one out of the three equivalent βs binds a nucleotide, and this β stabilizes γ in a particular orientation. Binding of the second ATP eventually shifts the stable orientation to one 120° ahead, but precisely when and how this happens is not yet clear. Logically, it is most likely that the binding of the second ATP immediately shifts the stable orientation (the minimum of the rotational potential for γ) forward, because some movement of γ would be required to promote the release of the hydrolysis products from the first site. Whether the binding of the second ATP alone, before the release of the hydrolysis products from the first site, moves the potential minimum 120° ahead remains to be seen. Complete 120° shift may require the product release, which, in addition to the ATP binding, is also expected to confer some energy to the F_1 motor. In this case, addition of sufficient ADP in the medium would stop the rotation at an intermediate angle. So far, we have been unable to observe pauses at intermediate angles, because the formation of the MgADP-inhibited form interferes with this kind of experiment. It is also possible that the ATP binding alone shifts the potential minimum by 120°, and the product release makes the minimum deeper.

Analysis of our stepping data suggests that, at least by the time the hydrolysis products are released (completion of a hydrolysis cycle), the rotational potential for γ is set up such that the minimum is 120° ahead of the previous minimum and the minimum is ~80 pN·nm deep (22). This depth determines the constant work output of this motor. The potential should be mostly downhill toward the minimum, because otherwise the rapid stepping cannot be explained.

TOWARD SINGLE-MOLECULE PHYSIOLOGY

We have shown that molecular rotations can be imaged continuously in real time, under an optical microscope, by the use of either a small tag such as a single fluorophore or a huge tag such as the actin filament. This implies that one can now image conformational changes, which necessarily involve reorientation (partial rotation), in a single protein machine at work. In fact, the rotation of F_1 is a conformational change over infinite angles. The stage has been set for 'single-molecule physiology,' where one observes and analyzes the behavior of single protein machines individually to elucidate the mechanism underlying their function.

A widely-used tag that is huge compared to protein machines is a plastic bead of a micrometer size. Individual steps of the linear motors kinesin (25) and myosin (26) have been measured with this tag. Consider an example where a plastic bead is attached at the tail end of an actin filament interacting with myosin on a glass surface (27). The bead is held with optical tweezers, and stepwise movement of actin caused by a myosin molecule is revealed as the displacement of the bead. A popular view is that the step arises entirely from a conformational change of myosin while it is bound to actin. Regardless of whether this is true or not, the experiment suggests that one can detect conformational changes in a protein molecule by attaching to it a plastic bead through a string of actin.

The measurement using a huge tag is precise, because it gives an intense optical signal. But a huge tag does not reveal which part of the molecule undergoes the conformational change. To answer this question, one could attach a small tag, a single fluorophore, at a desired site and measure its orientation as we have done in the detection of the axial rotation of an actin filament.

The huge tag we used to observe the rotation of F_1, the actin filament, is a smart probe in that it magnifies

the nanometer motion without the aid of sophisticated tools. An angular resolution of 30° is easily obtained even in direct observation by eye. The angle of 30° corresponds to the sliding of γ against the $\alpha_3\beta_3$ cylinder of only 0.5 nm. This tag gave us an additional bonus in that it allowed the measurement of force produced by the F_1 motor. The translational speed of the tip of the actin filament was tens of micrometers per second, more than an order of magnitude faster than the motion of a bead driven by linear motors. The fast motion accompanies a large enough frictional drag that counteracts the motor force. However, we were unable to measure the performance of the F_1 motor at no load. This can be achieved by the use of a small tag, as done in the single-fluorophore polarization measurement by Häsler et al. (20).

A huge probe also serves as a handle for manipulation. A plastic bead can easily be manipulated with optical tweezers (28, 29). By attaching a plastic bead to the actin filament bound to F_1, one could hopefully rotate F_1 in the reverse direction and synthesize ATP. Or, one could measure the rotational potential from the movement of the bead in the optical trap. Also, the use of single fluorophores is not limited to the detection of molecular orientations. For example, one can measure chemical reaction (ATP hydrolysis) in a single protein machine (30) simultaneously with the movement and force it produces (31). Applied to F_1, one could determine how the orientation of γ regulates the hydrolysis kinetics and how the bound nucleotides determine the rotational torque on γ. An optical microscope has hitherto served as one of the most important tools of cell biology. Now it opens up a new field of science, the single-molecule physiology. FJ

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REFERENCES

1. Tamm, S. L., and Tamm, S. (1974) Direct evidence for fluid membranes. *Proc. Natl. Acad. Sci. USA* **71,** 4589–4593
2. Tilney, L. G., and Inoué, S. (1987) Flagellar gyration and midpiece rotation during extension of the acrosomal process of *Thyone* sperm: how and why this occurs. *J. Cell Biol.* **104,** 407–415
3. Holmes, K. C., Popp, D., Gebhard, W., and Kabsch, W. (1990) Atomic model of the actin filament. *Nature (London)* **347,** 44–49
4. Nishizaka, T., Yagi, T., Tanaka, Y., and Ishiwata, S. (1993) Right-handed rotation of an actin filament in an in vitro motile system. *Nature (London)* **361,** 269–271
5. Sase, I., Miyata, H., Ishiwata, S., and Kinosita, K., Jr. (1997) Axial rotation of sliding actin filaments revealed by single-fluorophore imaging. *Proc. Natl. Acad. Sci. USA* **94,** 5646–5650
6. Kinosita, K., Jr., Itoh, H., Ishiwata, S., Hirano, K., Nishizaka, T., and Hayakawa, T. (1991) Dual-view microscopy with a single camera: real-time imaging of molecular orientations and calcium. *J. Cell Biol.* **115,** 67–73
7. Berg, H. C., and Anderson, R. A. (1973) Bacteria swim by rotating their flagellar filaments. *Nature (London)* **245,** 380–382
8. Jones, C. J., and Aizawa, S. (1991) The bacterial flagellum and flagellar motor: structure, assembly and function. *Adv. Microb. Physiol.* **32,** 109–172
9. DeRosier, D. J. (1998) The turn of the screw: the bacterial flagellar motor. *Cell* **93,** 17–20
10. Silverman, M., and Simon, M. (1974) Flagellar rotation and the mechanism of bacterial motility. *Nature (London)* **249,** 73–74
11. Noji, H., Yasuda, R., Yoshida, M., and Kinosita, K., Jr. (1997) Direct observation of the rotation of F_1-ATPase. *Nature (London)* **386,** 299–302
12. Boyer, P. D., and Kohlbrenner, W. E. (1981) The present status of the binding-change mechanism and its relation to ATP formation by chloroplasts. In *Energy Coupling in Photosynthesis* (Selman, B. R., and Selman-Reimer, S., eds) pp. 231–240, Elsevier, Amsterdam, The Netherlands
13. Mitchell, P. (1961) Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. *Nature (London)* **191,** 144–148
14. Boyer, P. D. (1993) The binding change mechanism for ATP synthase—some probabilities and possibilities. *Biochim. Biophys. Acta* **1140,** 215–250
15. Boyer, P. D. (1997) The ATP synthase—a splendid molecular machine. *Annu. Rev. Biochem.* **66,** 717–749
16. Abrahams, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994) Structure at 2.8 Å of F_1-ATPase from bovine heart mitochondria. *Nature (London)* **370,** 621–628
17. Duncan, T. M., Bulygin, V. V., Zhou, Y., Hutcheon, M. L., and Cross, R. L. (1995) Rotation of subunits during catalysis by *Escherichia coli* F_1-ATPase. *Proc. Natl. Acad. Sci. USA* **92,** 10964–10968
18. Zhou, Y., Duncan, T. M., and Cross, R. L. (1997) Subunit rotation in *Escherichia coli* F_0F_1-ATP synthase during oxidative phosphorylation. *Proc. Natl. Acad. Sci. USA* **94,** 10583–10587
19. Sabbert, D., Engelbrecht, S., and Junge, W. (1996) Intersubunit rotation in active F-ATPase. *Nature (London)* **381,** 623–625
20. Häsler, K., Engelbrecht, S., and Junge, W. (1998) Three-stepped rotation of subunits γ and ε in single molecules of F-ATPase as revealed by polarized, confocal fluorometry. *FEBS Lett.* **426,** 301–304
21. Kinosita, K., Jr., Yasuda, R., Noji, H., Ishiwata, S., and Yoshida, M. (1998) F_1-ATPase: a rotary motor made of a single molecule. *Cell* **93,** 21–24
22. Yasuda, R., Noji, H., Kinosita, K., Jr., and Yoshida, M. (1998) F_1-ATPase is a highly efficient molecular motor that rotates with discrete 120° steps. *Cell* **93,** 1117–1124
23. Livnah, O., Bayer, E. A., Wilchek, M., and Sussman, J. L. (1993) Three-dimensional structures of avidin and the avidin-biotin complex. *Proc. Natl. Acad. Sci. USA* **90,** 5076–5080
24. Nishizaka, T., Miyata, H., Yoshikawa, H., Ishiwata, S., and Kinosita, K., Jr. (1995) Unbinding force of a single motor molecule of muscle measured using optical tweezers. *Nature (London)* **377,** 251–254
25. Svoboda, K., Schmidt, C. F., Schnapp, B. J., and Block, S. M. (1993) Direct observation of kinesin stepping by optical trapping interferometry. *Nature (London)* **365,** 721–727
26. Finer, J. T., Simmons, R. M., and Spudich, J. A. (1994) Single myosin molecule mechanics: piconewton forces and nanometre steps. *Nature (London)* **368,** 113–119
27. Miyata, H., Yoshikawa, H., Hakozaki, H., Suzuki, N., Furuno, T., Ikegami, A., Kinosita, K., Jr., Nishizaka, T., and Ishiwata, S. (1995) Mechanical measurements of single actomyosin motor force. *Biophys. J.* **68,** 286s–290s
28. Svoboda, K., and Block, S. M. (1994) Biological applications of optical forces. *Annu. Rev. Biophys. Biomol. Struct.* **23,** 247–285
29. Harada, Y., Funatsu, T., Tokunaga, M., Saito, K., Higuchi, H., Ishii, Y., and Yanagida, T. (1998) Single molecule imaging and nanomanipulation of biomolecules. *Methods Cell Biol.* **55,** 117–128
30. Funatsu, T., Harada, Y., Tokunaga, M., Saito, K., and Yanagida, T. (1995) Imaging of single fluorescent molecules and individual ATP turnovers by single myosin molecules in aqueous solution. *Nature (London)* **374,** 555–559
31. Ishijima, A., Kojima, H., Funatsu, T., Tokunaga, M., Higuchi, H., Tanaka, H., and Yanagida, T. (1998) Simultaneous observation of individual ATPase and mechanical events by a single myosin molecule during interaction with actin. *Cell* **92,** 161–171