SINGLE-MOLECULE PHYSIOLOGY

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Single-Molecule Physiology under an Optical Microscope

Optical microscopy now enables us to image in real time, and also to manipulate, single protein (or RNA) machines that are 'alive' and at work. Studying the behavior of individual molecules is crucial to finding the mechanisms of molecular machines, because these machines operate stochastically and thus cannot be synchronized with each other in the rigorous sense. Single molecules often show apparent individualism, which also calls for individual detection. To study the individual behaviors, we propose the complementary use of huge and small tags. A tag that is huge ($\sim 10^2 \times$) compared to the size of a protein, *e.g.* a plastic bead, gives an intense optical signal that enables measurements at nanometer and millisecond precisions. A huge tag also serves as a handle for manipulations. A small tag such as a single fluorophore, on the other hand, can be attached at a desired site in a protein molecule and gives us specific information, *e.g.*, the spatial orientation of that particular site.

Under an optical microscope, we have observed individual behaviors of several molecular machines. Stepping rotation of the central subunit in a single molecule of F_1−ATPase has been videotaped (Fig. 1), and now we can discuss its detailed mechanism [1][2]. RNA polymerase has been shown to be a helical motor that precisely tracks the right-handed double helix of DNA (Fig. 2) [3], whereas myosin V has been shown to proceed as a left-handed spiral around an actin filament which is a right-handed double helix (Fig. 3) [4]. Huge tags such as micron-sized plastic beads also allow the manipulation of individual

molecules with, *e.g.*, optical or magnetic tweezers [3][4][5][6]. I personally believe that molecular machines operate by changing their conformations. Thus, detection of the conformational changes during function is our prime goal.

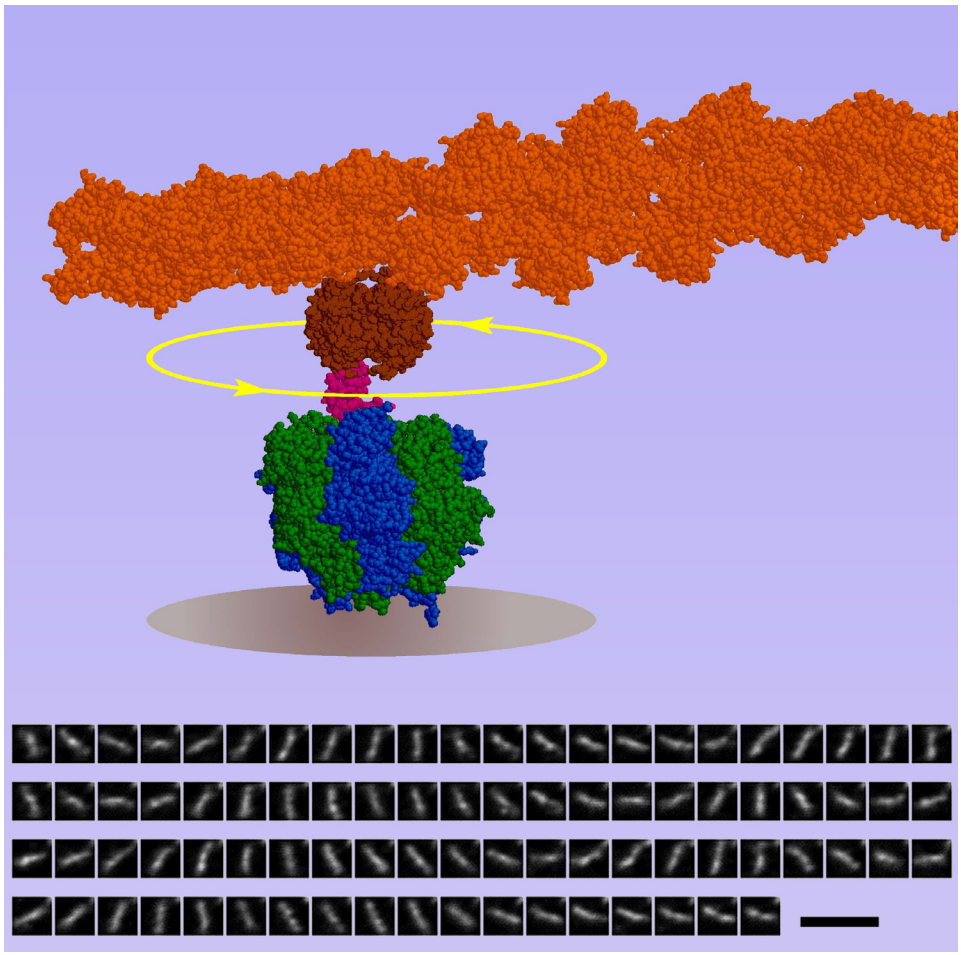

Fig. 1. *Rotation of F_1-ATPase, a rotary molecular motor* [9]. *Stator subunits (green and blue) were attached to the glass surface (gray), and a fluorescently stained actin filament (orange) was attached to the rotor subunit (magenta) through streptavidin (brown) that served as a glue. The gray disk measures ~22 nm. Sequential images at 33 ms intervals at the bottom shows counterclockwise rotation at 1.3 turns/s. Bar, 5 μm. The propeller rotation (rotation axis near the center of the filament) proves that the rotor subunit slides against the stator subunits over infinite angles.*

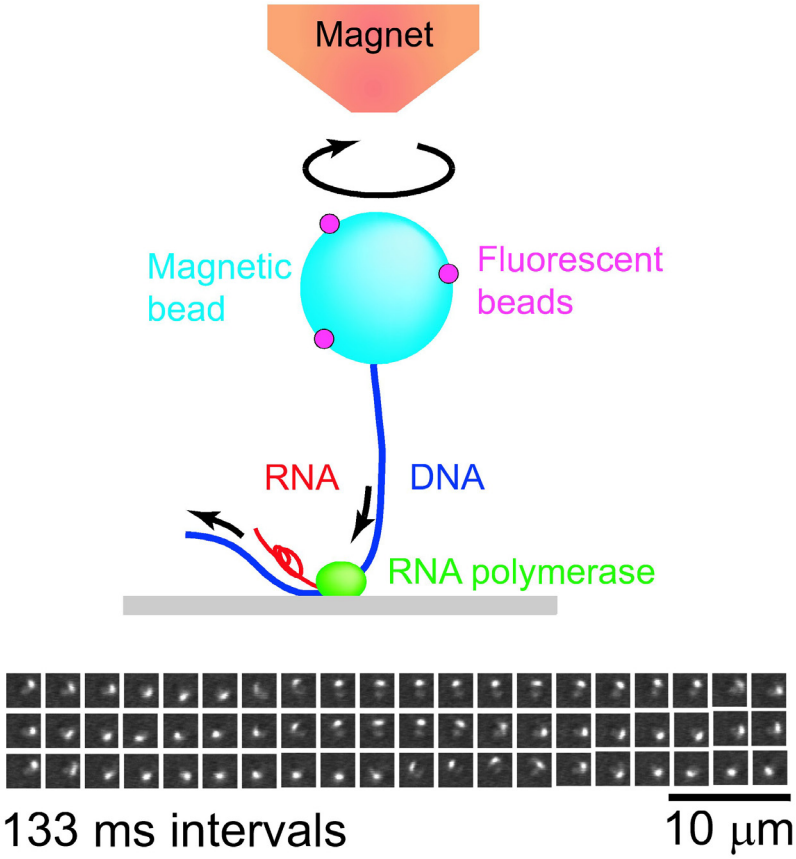

Fig. 2. *Rotation of DNA by RNA polymerase, a molecular machine that copies the genetic information on DNA onto a messenger RNA* [3]. *When a right-handed DNA helix threads through an RNA polymerase molecule fixed on a surface, rotation of the DNA as a right-handed screw is expected, as was indeed observed in the sequential images at the bottom (views from top in the cartoon). The magnetic bead attached to the tail end of DNA was pulled upward with a magnet to facilitate observation of rotation.*

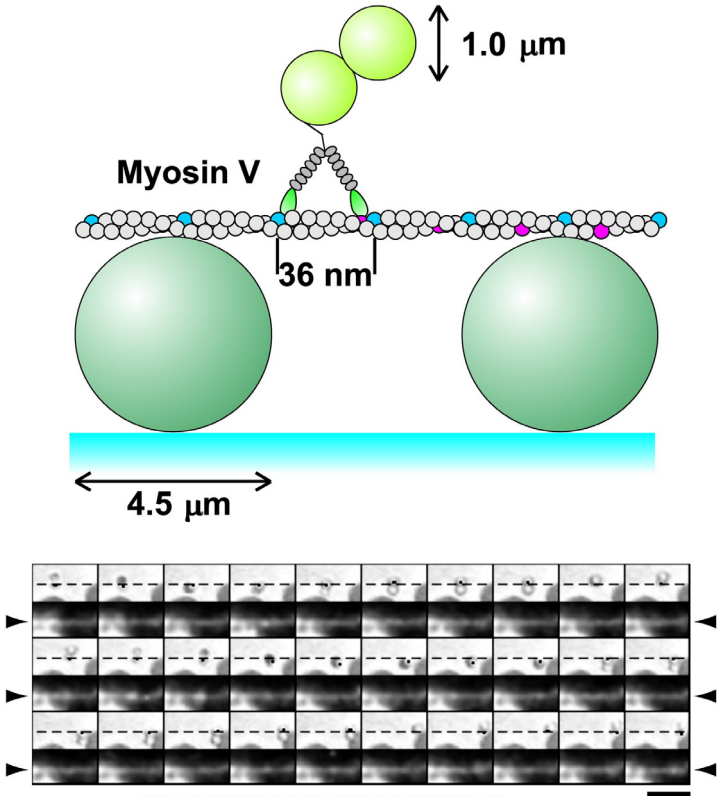

Fig. 3. *Spiral movement of myosin V around an actin filament* [4]. *A linear molecular motor myosin V moves along an actin filament, which is a right-handed double helix. To see if myosin V rotates around the filament, a bead duplex was attached to a single myosin molecule. An actin filament was suspended between two bigger beads, such that the bead-carrying myosin V could freely rotate around the filament. In the sequential image pairs (1 s intervals) at the bottom, upper rows show the bead duplex in a transmitted image, where a bead appears bright when it is closer to the observer and dark when it is away. Lower rows show that the actin filament, fluorescently stained, remained straight (arrow heads); the actin position is reproduced in upper images with a dashed line. Bar, 5 μm. As seen, the bead duplex moved as a left-handed spiral with a pitch of ~2.2 μm. This indicates that myosin V walks with a stride of slightly less than 36 nm, the actin helical repeat (landing between light-blue and magenta subunits in the cartoon).*

Stepping Rotation of F_1-ATPase

Splitting ATP into ADP and phosphate liberates free energy, but how this free energy can drive molecular machines in the cell is not fully understood yet. In general, ATP-driven molecular machines operate in three stages: (*i*) binding of ATP to a catalytic site, (*ii*) splitting of ATP in the catalytic site, and (*iii*) release of products (ADP and phosphate) from the catalytic site. We have shown experimentally that, at least for the rotary motor F_1-ATPase, stage (*i*) is the major force-producing step: most of mechanical work of this motor is done in stage (*i*). Stage (*iii*) also confers some force (work). But the splitting stage (*ii*), unlike the splitting reaction in solution, is more or less reversible and thus does not contribute much energy.

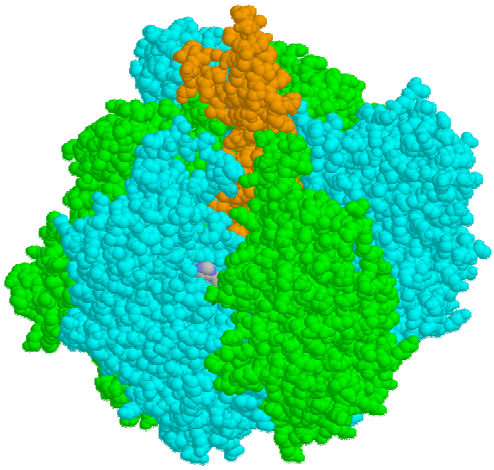

Fig. 4. *A crystal structure of F_1–ATPase [8]. The γ subunit shown in orange rotates in the cylinder made of three α (blue) and three β (green) subunits.*

F_1-ATPase

F_1-ATPase is a portion of the enzyme ATP synthase that synthesizes ATP from ADP and

phosphate using proton flow across a membrane as the energy source [7][1]. Isolated F_1-ATPase, which hydrolyzes ATP instead of synthesizing it, consists of $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$ subunits. In a crystal structure [8], the γ subunit is at the center and is surrounded by a cylinder made of $\alpha_3\beta_3$ subunits (Fig. 4). In 1997, we showed [9] that the isolated F_1-ATPase is a rotary motor, as proposed by Boyer [7], in which the central γ subunit rotates against the surrounding $\alpha_3\beta_3$ subunits when ATP is hydrolyzed in the three, catalytic β subunits. Reverse rotation of the γ subunit in ATP synthase, powered by the proton flow, is supposed to result in the ATP synthesis in the β subunits [7]. The F_1 rotation was visualized on a microscope by attaching an actin filament, a long rod that served as a marker, to the γ subunit while fixing the hexamer cylinder to a glass surface (Fig. 1) [8]. The sense of rotation was in accord with the crystal structure of F_1 [8] which hinted sequential ATP hydrolysis on the three β subunits. Imaging F_1 rotation has so far revealed the following mechanical properties of this molecular motor: (*a*) the rotation consists of discrete 120° steps each driven by hydrolysis of one ATP molecule [10][11]; (*b*) the mechanical work done in each step is constant and is 80-90 pN·nm, which is comparable to the free energy obtained by hydrolysis of one ATP molecule [10]; (*c*) the torque (rotary force) of this motor is approximately constant against the stepping angle [1]. Points (*a*) and (*b*) imply that the energy-conversion efficiency of this ATP-driven molecular motor can reach ~100% [10]. Precise correspondence between the hydrolysis reaction and mechanical rotation, however, could not be established in these earlier studies.

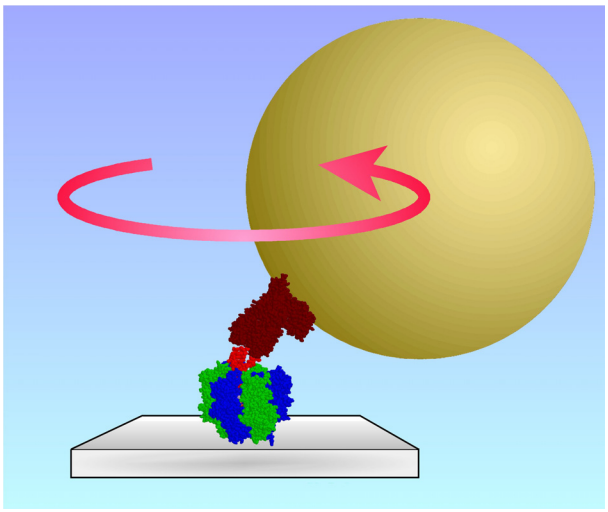

Fig. 5. *Imaging F_1 rotation through a gold bead* [2]. *The β (green) subunits were fixed on a glass surface, and a 40-nm gold bead was attached to the central γ subunit (red) through streptavidin and BSA (brown) that served as glue. When the bead was attached obliquely as shown in the figure, rotation of the γ subunit resulted in a circular movement of the bead image. The rotation angle was estimated from the circular trajectory of the bead movement.*

Substeps and the Mechanism of Rotation

Recently, we have attached a 40-nm colloidal gold bead to the γ subunit and imaged its rotation at 8,000 frames per s [2] (see Fig. 5). At a saturating ATP concentration of 2 mM, the motor rotated at 130 revolutions per second (~8,000 revolutions per minute at 23°C for the motor obtained from a thermophilic bacterium). The 120° steps were clearly resolved even at this full speed, and the speed during stepping exceeded 100,000 revolutions per minute. At lower speeds, the motor showed distinct ~90° and ~30° substeps, each taking only a fraction of a millisecond (Fig. 6). Analysis of the substep kinetics has suggested the scheme in Fig. 7a: the 90° substep is driven by ATP binding, and the 30° substep by product release.

Between 90° and 30° substeps are two ~1 ms reactions that are mechanically silent, which may correspond to hydrolysis and release of a product. The substeps are not resolved at 2 mM ATP, because a 90° substep immediately follows a 30° substep.

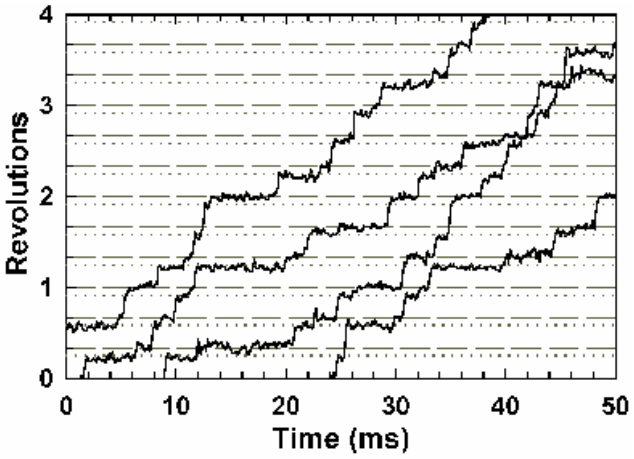

Fig. 6. *Substeps in the rotation of F_1-ATPase at 20 μM ATP* [2]. *All curves are continuous, the latter curves being shifted to save space. Long dashed lines are drawn at intervals of 120°, and dotted lines are drawn 30° below the dashed lines.*

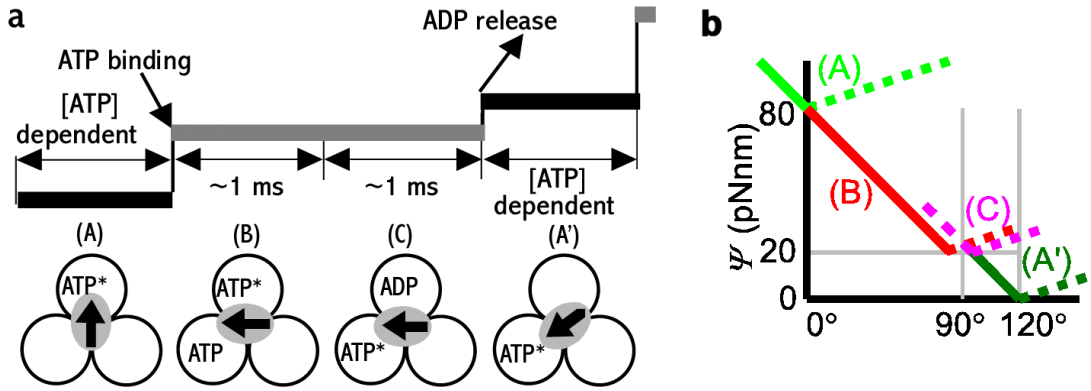

Fig. 7. *Kinetics of substeps* ***(a)*** *and deduced rotational potentials* ***(b)***. *ATP* stands for ATP or ADP + phosphate; ADP may by phosphate or ADP + phosphate. Colored lines in* ***b*** *represent the angle-dependent potential energy for γ rotation.* ***(A)-(A')*** *are potential energies for the corresponding chemical states in* ***a***. *Adapted from* [2].

From the scheme in Fig. 7a and the previous observation that the torque of this motor is angle-independent, we can deduce the potential energy for the γ rotation as shown in Fig. 7b. In the one-nucleotide state (A) in Fig. 7a, the potential energy is minimal at the equilibrium angle, which is taken as 0°. Binding of a second ATP (B) produces a potential that is downhill towards the angle 90° ahead, with a constant slope indicating a constant torque. Upon the release of the last hydrolysis product, the potential energy returns to that in (A) except for the 120° difference; the potential in (A') is again linearly downhill at least over the interval between 90° and 120°.

That ATP binding drives the 90° substep implies, by the law of action and reaction, that the reversal of a 90° substep (B→A in Fig. 7a) would reduce the affinity for ATP of the β subunit on the left of the arrow in Fig. 7a. The magnitude of the affinity decrease can be estimated from the potential diagram in Fig. 7b, and is more than 2,000,000 fold [2]. Likewise, reversal of a 30° substep (A'→C) accompanies an increase of >100 fold in the affinity for ADP of the β subunit on the right of the arrow. These affinity changes can account for ATP synthesis when the γ subunit is forced to rotate clockwise in Fig. 7a (by the action of proton flow through ATP synthase). Starting from A' in Fig. 7a, the β subunit on the right of the arrow will pick up ADP from the medium when the γ subunit turns 30° clockwise. Further clockwise rotation by 90° will reduce the affinity for the previously synthesized ATP on the left of the arrow, and this ATP is released into the medium. This scheme indicated by our experiment is an embodiment of the binding change mechanism for ATP synthesis proposed by Boyer many years ago [7].

ATP and [ADP + phosphate] are in equilibrium in the catalytic site, and thus synthesis or hydrolysis of ATP on the enzyme do not accompany much change in free energy

(synthesis and hydrolysis are basically reversible on the enzyme). Myosin also hosts ATP and [ADP + phosphate] in equilibrium. Many ATP-driven molecular machines may adopt this strategy: by binding ATP tightly, they stabilize the ATP form such that its free energy is comparable with that of ADP + phosphate. Then, much of the free-energy drop must occur in the ATP binding step. An efficient molecular machine should convert this maximal energy into work. The role of hydrolysis *per se* is mainly to reset the machine for the next round of cycle, by separating ATP into two entities and thereby allowing them to dissociate easily. For the completely reversible F_1 motor, hydrolysis may well play an additional role of correctly determining the sense of rotation (through coupling with a small amount of rotation) [1][2].

F_1-ATPase tends to fall in a dormant state when it binds MgADP tightly (MgADP inhibition). Rotation then stops, but, in the presence of ATP, the motor eventually wakes up and resumes continuous rotation, until it falls again into the inhibited state [1][7]. The orientation of the γ subunit in the inhibited state is close to the angle after a ~90° substep [12], which is consistent with the idea that the inhibition is caused by the failure in releasing ADP. Release of phosphate may also contribute to the production, or control, of rotary torque [13].

Rotation of F_1-ATPase is also powered by GTP or ITP, but not by UTP or CTP [14]. Mechanical characteristics of GTP- or ITP-driven rotation appear to be quite similar to those of ATP-driven rotation. It thus seems that the protein contains in it a precisely preset rotary mechanism, which a nucleotide either enables to proceed or fails to activate.

In the lecture, I will also present results of recent experiments, including an attempt at imaging binding and release of ATP in single F_1-ATPase molecules, and discuss a model of F_1 motor. Also, if time allows, I will discuss a general principle of how one could design an efficient molecular machine.

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