IX-D Single-Molecule Physiology

A single molecule of protein (or RNA) enzyme acts as a machine which carries out a unique function in cellular activities. To elucidate the mechanisms of various molecular machines, we need to observe closely the behavior of individual molecules, because these machines, unlike man-made machines, operate stochastically and thus cannot be synchronized with each other. By attaching a tag that is huge compared to the size of a molecular machine, or a small tag such as a single fluorophore, we have been able to image the individual behaviors in real time under an optical microscope. Stepping rotation of the central subunit in a single molecule of F_1-ATPase has been videotaped, and now we can discuss its detailed mechanism. RNA polymerase has been shown to be a helical motor that rotates DNA during transcription. Single-molecule physiology is an emerging field of science in which one closely watches individual, 'live' protein/RNA machines at work and examines their responses to external perturbations such as pulling and twisting. I personally believe that molecular machines operate by changing their conformations. Thus, detection of the conformational changes during function is our prime goal. Complementary use of huge and small tags is our major strategy towards this end.

IX-D-1 Resolution of Distinct Rotational Substeps by Submillisecond Kinetic Analysis of F_1-ATPase

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The enzyme F_1-ATPase has been shown to be a rotary motor in which the central γ-subunit rotates inside the cylinder made of $\alpha_3\beta_3$ subunits. At low ATP concentrations, the motor rotates in discrete 120° steps, consistent with sequential ATP hydrolysis on the three β-subunits. The mechanism of stepping is unknown. Here we show by high-speed imaging that the 120° step consists of roughly 90° and 30° substeps, each taking only a fraction of a millisecond. ATP binding drives the 90° substep, and the 30° substep is probably driven by release of a hydrolysis product. The two substeps are separated by two reactions of about 1 ms, which together occupy most of the ATP hydrolysis cycle. This scheme probably applies to rotation at full speed (~130 revolutions per second at saturating ATP) down to occasional stepping at nanomolar ATP concentrations, and supports the binding-change model for ATP synthesis by reverse rotation of F_1-ATPase.

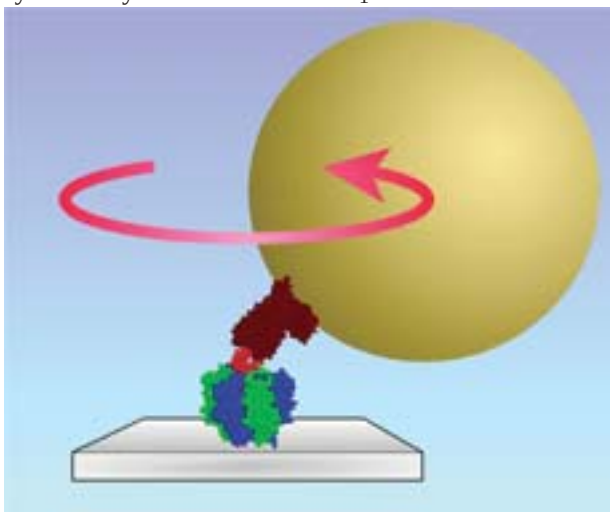

Figure 1. Imaging F_1 rotation through a gold bead. The cylinder made of three α (blue) and three β (green) subunits was fixed on a glass surface, and a 40-nm gold bead was attached to the central γ subunit (red) through streptavidin and BSA (brown) that served as glue. When the bead was attached obliquely 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.

IX-D-2 Purine but Not Pyrimidine Nucleotides Support Rotation of F_1-ATPase

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The binding change model for the F_1-ATPase predicts that its rotation is intimately correlated with the changes in the affinities of the three catalytic sites for nucleotides. If so, subtle differences in the nucleotide structure may have pronounced effects on rotation. Here we show by single-molecule imaging that purine nucleotides ATP, GTP, and ITP support rotation but pyrimidine nucleotides UTP and CTP do not, suggesting that the extra ring in purine is indispensable for proper operation of this molecular motor. Although the three purine nucleotides were bound to the enzyme at different rates, all showed similar rotational characteristics: counterclockwise rotation, 120° steps each driven by hydrolysis of one nucleotide molecule, occasional back steps, rotary torque of ~40 piconewtons (pN)·nm, and mechanical work done in a step of ~80 pN·nm. These latter characteristics are likely to be determined by the rotational mechanism built in the protein structure, which purine nucleotides can energize. With ATP and GTP, rotation was observed even when the free energy of hydrolysis was –80 pN·nm/molecule, indicating ~100% efficiency. Reconstituted F_oF_1-ATPase actively translocated protons by hydrolyzing ATP, GTP, and ITP, but CTP and UTP were not even hydrolyzed. Isolated F_1 very slowly hydrolyzed UTP (but not CTP), suggesting possible uncoupling from

rotation.

IX-D-3 Direct Observation of DNA Rotation during Transcription by *Escherichia coli* RNA Polymerase

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Helical filaments driven by linear molecular motors are anticipated to rotate around their axis, but rotation consistent with the helical pitch has not been observed. 14S dynein and non-claret disjunctional protein (ncd) rotated a microtubule more efficiently than expected for its helical pitch, and myosin rotated an actin filament only poorly. For DNA-based motors such as RNA polymerase, transcription-induced supercoiling of DNA supports the general picture of tracking along the DNA helix. Here we report direct and real-time optical microscopy measurements of rotation rate that are consistent with high fidelity tracking. Single RNA polymerase molecules attached to a glass surface rotated DNA for .100 revolutions around the right-handed screw axis of the double helix with a rotary torque of > 5 pN nm. This real-time observation of rotation opens the possibility of resolving individual transcription steps.

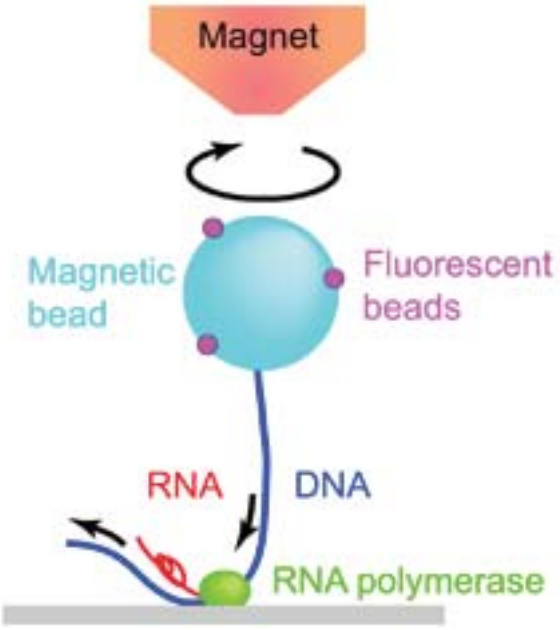

Figure 1. Observation of DNA rotation by RNA polymerase. The magnetic bead was pulled upwards by a disk-shaped neodymium magnet, to which a conical iron piece was attached to enhance the magnetic force. Magnetization was vertical and did not prevent bead rotation. Daughter fluorescent beads served as markers of rotation.