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Designing a mutant F:\textsubscript{\textgamma}ATPase for easy and rapid single molecule analysis.

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F:\textsubscript{\textgamma}ATPase is a part of ATP synthase and well known as an ATP-driven rotary motor. Subunit composition of F:\textsubscript{\textgamma}ATPase is $\alpha\beta\gamma\delta\epsilon$, in which the $\epsilon$-subunits act as a rotor. The rotation of single-molecule F:\textsubscript{\textgamma}ATPase has been visualized under microscope as the rotation of avidin-coated actin filament or bead. These rotation probes are attached to the biotin, which is chemically introduced to the thiol group of cysteine residues within the $\gamma$-subunit. In this study, we designed a mutant F:\textsubscript{\textgamma}(F:\textsubscript{\textgamma}-avi), whose $\beta$-subunit has a His10-tag at N-terminus and $\gamma$-subunit has an avi-tag sequence between Ala-108 and Ser-109 residues. Avi-tag is a 15-residue long peptide and a lysine residue within the tag is biotinylated in E. coli by biotin-ligase. We could observe the rotation of bead on Ni-NTA coated glass using cell-free extract of E. coli expressing F:\textsubscript{\textgamma}-avi. In this system, neither purification nor chemical modification is required before rotation assay. Because biotinylated of $\gamma$-subunit does not involve cysteine residue in this system, we will be able to utilize cysteine residue for other purposes, such as fluorescence modification or disulfide bond formation. We will report basic properties of rotation of F:\textsubscript{\textgamma}-avi, including torque and $k_{\text{m}}$ for ATP. We are also trying to introduce second avi-tag to $\gamma$-subunit to improve the system.

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Single-molecule analysis of F:\textsubscript{\textgamma}motor loaded with nonhydrolyzable substrate

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F:\textsubscript{\textgamma}ATPase ($\alpha\beta\gamma\delta\epsilon\zeta$) is a rotary motor protein in which an inner subunit ($\gamma$) rotates against a surrounding cylinder ($\alpha\beta\delta\epsilon\zeta$). It makes a 120-degree step rotation upon one ATP hydrolysis. Previous studies revealed that the 120-degree step consists of 80- and 40-degree sub-steps. 80-degree sub-step is initiated by ATP binding, 40-degree sub-step is presumably products release, and the torque is not generated by hydrolysis energy. This suggests that the ATP-binding is the major energy releasing step for torque generation in the mechaenochemical coupling of F:\textsubscript{\textgamma}ATPase. However exact verification remains to be done. To access this task, we conduct single-molecule analysis of F:\textsubscript{\textgamma} rotation using a nonhydrolyzable substrate, adenosine 5'- ([\beta\gamma-imido]triphosphate (AMP-PNP). When buffer was exchanged to replace ATP with AMP-PNP, F:\textsubscript{\textgamma} molecules paused the rotation. Statistical analysis showed that F:\textsubscript{\textgamma} pauses at 80-degree forward from ATP binding position, which well agrees with the model for catalysis and rotation. We will show what amount of work F:\textsubscript{\textgamma} does during AMP-PNP binding process to clarify the above question.

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Does the forcible rotation of F:\textsubscript{\textgamma} motor enhance ATP hydrolysis?

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F:\textsubscript{\textgamma} motor hydrolyzes three ATP molecules to make a rotation of the central $\gamma$ subunit. In a previous study, we had shown that mechanical reverse rotation of F:\textsubscript{\textgamma} motor leads the reverse reaction, ATP synthesis. The determined coupling efficiency was very high; mostly three ATP molecules were generated per reverse turn. Here, a simple question arises, "does forcible forward rotation enhance ATP hydrolysis reaction?" To solve this task, we conducted the experiment similar to the previous one. A rotating molecule was enclosed in a 6 femtoliter chamber, with 500 nM ATP which contains 1800 molecules of ATP in a 6 femtoliter. Then, the motor was forcibly rotated in the forward direction for 300 turns using magnetic tweezers. The consumption of ATP during the forcible rotation was calculated from the rotation velocity of the motor released from the magnetic tweezers; in such low ATP concentrations, the rotary velocity of F:\textsubscript{\textgamma} motor is proportional to ATP concentration, therefore, the ATP consumption can be determined from the decrement of the rotary velocity after forcible rotation. When rotated at 5 Hz, the efficiency was very high nearly 100 % while a low efficiency was obtained at 10 Hz. Thus, it was shown that the forcible forward rotation enhances ATP hydrolysis reaction, at least ATP binding step, and the efficiency depends on the rotation velocity.

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Development of the Single Molecule Imaging System of the F:\textsubscript{\textgamma} Motor

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F:\textsubscript{\textgamma} motor of F:\textsubscript{\textgamma}\textsubscript{\textupsilon}\textsubscript{\textbeta}\textsubscript{\textomega}\textsubscript{\textalpha}ATP synthase (F:\textsubscript{\textgamma}\textsubscript{\textupsilon}\textsubscript{\textbeta}) acts as the proton channel that converts electrochemical energy of the proton gradient into mechanical energy. It is important in biology and physics to study the rotary mechanism of F:\textsubscript{\textgamma} motor. However, direct observation of the rotation of F:\textsubscript{\textgamma} motor driven by proton motive force has not been achieved and the rotary mechanism of F:\textsubscript{\textgamma} motor is yet unknown. So, to elucidate its rotary mechanism, we developed the single molecule imaging system (Ueno et al., Annual Meeting of the BSJ 2005). This system is composed of the planar membrane system that can control membrane potential and the direct observation system of F:\textsubscript{\textgamma}. And this time, we generated the new F:\textsubscript{\textgamma}\textsubscript{\textupsilon}\textsubscript{\textbeta} mutants which had Avitag (15-amino acid sequence for biotinylation in vivo) or Cysteine (for in vitro biotinylation) at the C-terminus of the F:\textsubscript{\textgamma}-b subunit and His-tag at the N-terminus of the F:\textsubscript{\textgamma}-c subunit to detect rotation. These mutants exhibited the substantial proton pumping and ATPase activity that were roughly similar to those of wild type after purification and reconstitution into liposomes. These results indicate that these mutants maintain activity and coupling function as same as wild type. Then, we tried the rotation assay using these mutants. These mutants showed the ATP-driven rotation in detergent, indicating that these introduced tags are in appropriate position to transmit the rotary torque to the rotation-probe. Now, we try to detect the rotation of F:\textsubscript{\textgamma} motor isolated from these mutants in the planar membrane by this system.