Single Molecule Energetics of F_1-ATPase Motor

Eiro Muneyuki,* Takahiro Watanabe-Nakayama,† Tetsuya Suzuki,† Masasuke Yoshida,† Takayuki Nishizaka,‡§ and Hiroyuki Noji§¶

*Department of Physics, Faculty of Science and Technology, Chuo University, Tokyo, Japan; †Chemical Resources Laboratory, Tokyo Institute of Technology, Yokohama, Japan; ‡Department of Physics, Gakushuin University, Tokyo, Japan; §Precursory Research for Embryonic Science and Technology (PRESTO), Japan Science and Technology Agency, Saitama, Japan; and ¶The Institute of Scientific and Industrial Research (ISIR) Osaka University, Osaka, Japan

ABSTRACT Motor proteins are essential in life processes because they convert the free energy of ATP hydrolysis to mechanical work. However, the fundamental question on how they work when different amounts of free energy are released after ATP hydrolysis remains unanswered. To answer this question, it is essential to clarify how the stepping motion of a motor protein reflects the concentrations of ATP, ADP, and P_i in its individual actions at a single molecule level. The F_1 portion of ATP synthase, also called F_1-ATPase, is a rotary molecular motor in which the central γ-subunit rotates against the $\alpha_3\beta_3$ cylinder. The motor exhibits clear step motion at low ATP concentrations. The rotary action of this motor is processive and generates a high torque. These features are ideal for exploring the relationship between free energy input and mechanical work output, but there is a serious problem in that this motor is severely inhibited by ADP. In this study, we overcame this problem of ADP inhibition by introducing several mutations while retaining high enzymatic activity. Using a probe of attached beads, stepping rotation against viscous load was examined at a wide range of free energy values by changing the ADP concentration. The results showed that the apparent work of each individual step motion was not affected by the free energy of ATP hydrolysis, but the frequency of each individual step motion depended on the free energy. This is the first study that examined the stepping motion of a molecular motor at a single molecule level with simultaneous systematic control of ΔG_{ATP}. The results imply that microscopically defined work at a single molecule level cannot be directly compared with macroscopically defined free energy input.

INTRODUCTION

In many biological ATP-driven motor proteins, stepping motion (linear or rotary) is observed at a single molecule level. In these cases, the free energy liberated by ATP hydrolysis (ΔG_{ATP}) is the energy input, and the mechanical step motion is the apparent output. This gives rise to an intriguing question: how does the change in ΔG_{ATP} affect the stepping motion? For example, how do molecular motors behave at a very low ΔG_{ATP}? As free energy is a macroscopically defined quantity and stepping motion of a single molecule is a microscopic feature, the above question is asking how to relate the law of the macroscopic world to observation in the microscopic world.

At first, the following may be considered as the possible behaviors:

1. The stepping velocity depends on ΔG_{ATP}; therefore, there is a change in the mechanical work done by the individual step motion.
2. The motor generates a step motion of a fixed size and velocity, irrespective of the ΔG_{ATP} input. However, when the chemical energy input (ΔG_{ATP}) becomes lower than the mechanical energy output in one step, it simply stops all step motions except those required for thermal fluctuation.
3. It might be possible that when one ATP is hydrolyzed, constant mechanical work is derived irrespective of the ΔG_{ATP}. However, the frequency of the forward step motion decreases as ΔG_{ATP} decreases and the back step increases due to the reverse reaction.

However, each of the three possibilities stated above has its limitations. In the case of possibility 1 or 2, a single motor molecule has to detect ΔG_{ATP}, which is a function of [ATP], [ADP] and [P_i] in a bulk medium. In addition, the mechanism of changing or stopping the stepping motion in response to ΔG_{ATP} remains unclear. Alternatively, in the case of possibility 3, it might be possible that the constant mechanical work exceeds the ΔG_{ATP} input; this seems to violate the law of thermodynamics.

The simple abovementioned question on the action of molecular motors has remained unanswered due to many experimental difficulties. For example, myosin II easily detaches from an actin filament, preventing the continuous observation of a single molecule under different conditions. Myosin V or kinesin's step motions yield a relatively low mechanical energy. Moreover, in the presence of a high concentration of ADP or P_i, which is required for controlling ΔG_{ATP}, carrying out a single molecule motility assay for these linear motors is quite difficult. This study aims to

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Address reprint requests to Eiro Muneyuki, Dept. of Physics, Faculty of Science and Technology, Chuo University, 1-13-27 Kasuga, Bunkyo-ku, Tokyo 112-8551, Japan. Tel.: 81-3-3817-1769; Fax: 81-3-3817-1792; E-mail: emuneyuk@phys.chuo-u.ac.jp.

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experimentally answer the above question by using a rotary molecular motor, namely, F_1-ATPase.

F_1-ATPase, a water-soluble portion of ATP synthase (1), is a rotary molecular motor in which the central γ-subunit rotates against the $\alpha_3\beta_3$ cylinder, hydrolyzing ATP (2). The rotation is stepwise. When one ATP molecule is hydrolyzed, the γ-subunit makes a 120° step (anticlockwise, viewed from the F_o side) (3). The structure of this motor is highly stable, giving rise to a continuous, processive stepping rotation. In addition, the torque generated is considerably high (2–6). These features are ideal to explore the relationship between free energy input and mechanical work output. The only drawback is that this motor protein is severely inhibited by ADP. This ADP inhibition, also known as ADP-Mg inhibition, is not a simple product inhibition. Extensive kinetic analyses of ADP-Mg inhibition have lead to the conclusion that when ADP-Mg is formed during ATP hydrolysis or if it binds from the external medium, then the enzyme enters an inhibited state, which barely releases the bound ADP-Mg (7–9). Due to this specific inhibition, it has been practically impossible to observe continuous rotations while controlling ΔG_{ATP} by adding high concentrations of ADP. To overcome this problem, we introduced several mutations in $\alpha_3\beta_3\gamma$, the minimal subcomplex of F_1 that shows ATP-catalyzed rotation (Fig. 1). Using the mutant, termed as GT mutant, we investigated the relationship between ΔG_{ATP} and the stepping motion against viscous load. This is the first report in which the stepping motion of a molecular motor was examined at a single molecule level while ΔG_{ATP} was systematically controlled.

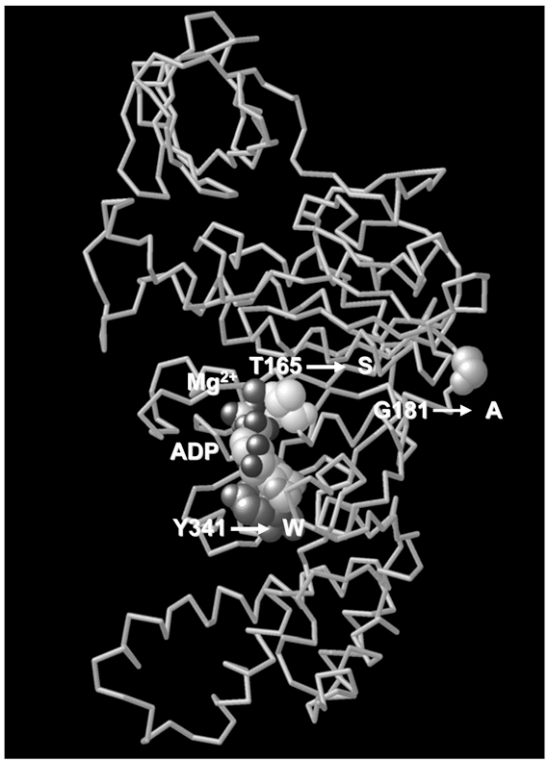

FIGURE 1 Mutations specifically introduced in the β-subunit of the GT mutant. Mutations common to the previous mutants used in the rotation assay are not shown.

MATERIALS AND METHODS

Protein

The mutant of the $\alpha_3\beta_3\gamma$ subcomplex resistant to ADP-Mg inhibition was derived from F_1-ATPase of a thermophilic *Bacillus* PS3 by introducing the following mutations: β-G181A (10) and β-T165S (11) for minimizing ADP-Mg inhibition; α-C193S, γ-S107C, and γ-I210C for specific biotinylation of the γ-subunit (12); β-His_{10} at the amino terminus (2); α-W463F and β-Y341W (13) (Fig. 1). The mutant was named as GT mutant. In this manuscript, we call α-W463F / β-Y341W mutant as wild-type F_1. The protein was expressed and purified by the conventional method described in Dou et al. (14). The purified enzyme hardly retained bound nucleotides (<0.05 mol/mol of enzyme).

ATP hydrolysis was measured as a decrease in the absorbance of NADH at 340 nm at 25°C by using an ATP-regenerating system. Since the GT mutant showed a lag phase in its ATPase activity, the maximum rate of ATP hydrolysis was determined several minutes after the start of the reaction.

Rotation assay

Carboxy-modified polystyrene beads (0.35 μm in diameter; Polysciences, Warrington, PA) were modified with 0.5 $mg{\cdot}ml^{-1}$ biotin-X cadaverine (Molecular Probes, Eugene, OR) and 2.5 $mg{\cdot}ml^{-1}$ 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Pierce, Rockford, IL) at room temperature for 2 h in dark; unreacted biotin was removed by centrifugation. Next, 1 mg ml^{-1} NeutrAvidin or streptavidin (Sigma, St. Louis, MO) was added, and the excess avidin was washed off. Biotin (BiotinPEAC$_5$maleimide; Dojindo, Kumamoto, Japan) was coupled to Cys residues in the γ-subunit of F_1 by a 30-min incubation at room temperature at 1~8 μM of F_1 at F_1/biotin = 1:2. To observe the rotation, ~100 pM F_1 in buffer A was infused into a flow chamber that comprised of two glass coverslips. The volume of the chamber was ~15 μl.

F_1 tended to attach to the glass surface in a nonspecific manner. After 2 min, the chamber was washed with 20 mM MOPS-KOH, 10 mM $MgCl_2$, 10 $mg{\cdot}ml^{-1}$ BSA, 0.1 M potassium phosphate at pH 7, and the avidin-coated beads at 0.1% concentration were then infused. Avidin on the beads specifically attached to the γ-subunit in a single F_1, while $\alpha_3\beta_3$ was immobilized on the surface. Stepping between $\alpha_3\beta_3$ and γ was visualized as bead displacement. The buffer system used for the rotation assay contained 20 mM MOPS-KOH, 10 mM $MgCl_2$, 10 $mg{\cdot}ml^{-1}$ BSA, 0.1 M potassium phosphate at pH 7, and 2 or 10 μM ATP. To change the ΔG_{ATP}, ~40 or 60 μl of the buffer containing different concentrations of ADP was infused at least twice.

In this study, [ATP] was fixed at 2 or 10 μM, [P_i] was fixed at 0.1 M, and [ADP] was varied from 0.2 to 1000 μM to change ΔG_{ATP}. The reason that we chose only [ADP] as a variable to control ΔG_{ATP} was that we wanted to maintain ionic strength (nearly) constant by keeping high [P_i] constant. Change in the ionic strength may cause change in friction between beads probe and glass surface. Addition of other salts to adjust the ionic strength might change enzymatic activity of F_1-ATPase. The stock solution of ADP was treated with hexokinase and glucose to remove any contaminating ATP. The level of ATP contamination after treatment was measured using a luciferase assay and was found to be <0.05%. ΔG_{ATP} was calculated by the following equation according to Nicholls (15).

$$\Delta G_{ATP} = \Delta G^{O}_{ATP} + 2.3\,RT \log \frac{[\sum ADP]\,[\sum Pi]}{[\sum ATP]},$$

at pH 7.0, 10 mM Mg^{2+}.