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TRANSPORT ATPASES: STRUCTURE, MECHANISM AND RELEVANCE TO MULTIPLE DISEASES

Abundance of *Escherichia coli* F_1-ATPase molecules observed to rotate via single-molecule microscopy with gold nanorod probes

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Abstract The abundance of *E. coli* F_1-ATPase molecules observed to rotate using gold nanorods attached to the γ-subunit was quantitated. Individual F_1 molecules were determined to be rotating based upon time dependent fluctuations of red and green light scattered from the nanorods when viewed through a polarizing filter. The average number of F_1 molecules observed to rotate in the presence of GTP, ATP, and without nucleotide was ~50, ~25, and ~4% respectively. In some experiments, the fraction of molecules observed to rotate in the presence of GTP was as high as 65%. These data indicate that rotational measurements made using gold nanorods provide information of the F_1-ATPase mechanism that is representative of the characteristics of the enzyme population as a whole.

Keywords F_1-ATPase · Gold nanorods · Plasmon resonance · Molecular motors · Single molecule microscopy

Introduction

The F_1F_o ATP synthase is composed of a transmembrane F_o complex and extrinsic membrane F_1 complex that work together to couple the energy from a nonequilibrium transmembrane proton gradient into the synthesis of ATP from ADP and phosphate. Both complexes are rotary molecular motors that use a common drive shaft composed of the γ and ε subunits. The F_1-ATPase driven rotation and F_o-proton gradient driven rotation of the ε and γ subunits occur in opposite directions (Noji et al. 1997; Borsch et al. 2002).

The F_1-ATPase can be purified from F_o and the membrane, and studied as a soluble protein. Hydrolysis of ATP occurs at the interface of each of the three $\alpha\beta$ subunit heterodimers that form a ring around the coiled-coil domain of the γ subunit (Abrahams et al. 1994). When F_1 is attached to a Ni-NTA coated microscope slide via histidine tags on α or β subunits, and a visible probe is attached to the γ-subunit, single molecules of the γ-subunit can be observed to rotate via microscopy (Noji et al. 1997). In the presence of saturating amounts of substrate, the F_1 γ-subunit rotates in 120° step increments that correspond to the hydrolysis of one ATP per event (Yasuda et al. 1998).

When fluorescent actin filaments were used as a reporter group to visualize rotation, Noji et al. (1997) observed less than 2% of F_1 from the thermophilic bacterium *PS3* to rotate, whereas Adachi et al. (2000) observed 10%. About 5% of *E. coli* F_1-ATPase molecules examined using actin filaments were observed to rotate (Panke et al. 2000). Actin filaments are very large with respect to F_1, which increases the potential for these probes to interfere significantly with the ability of the motors to drive rotation. Rotation has also been detected using nanospheres that varied from 40 nm to 1 μm in diameter as probes. These measurements rely on the ability to detect the eccentricity of γ subunit rotation through calculation of the centriod movement of a diffraction-limited 250 nm diameter spot of green light scattered from the nanosphere, and measured with a video

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or digital camera (Yasuda et al. 2001). The fraction of molecules observed to rotate increased to about 10% for *E. coli* F_1 through the use of nanospheres as rotation probes (Nakanishi-Matsui et al. 2006). Although the causes of such a low yield of rotating molecules remain unclear, this observation has been explained by postulating that only 10% of the molecules in an F_1 preparation are active, and that the active motors hydrolyze ATP at a rate 10-fold higher than would be expected from bulk measurements (Nakanishi-Matsui et al. 2006). However, because such a small percentage of the population of molecules has been observed to rotate, it has been difficult to conclude that the measurements made that characterize rotation are representative of the enzyme population as a whole.

Spetzler et al. (2006) recently reported a new method to detect F_1-ATPase rotation using 35×75 nm gold nanorod probes viewed through a polarizing filter. The nanorods scatter green and red light intensely when the long axis is perpendicular and parallel to the plane of a polarizing filter, respectively (Raschke et al. 2003; Sonnichsen and Alivisatos 2005), such that F_1-ATPase-driven rotation of these probes results in a predictable color change. The large dynamic range of intensity fluctuations of the red and green light as a function of the angular position between the nanorod and the polarizing filter provides a much more sensitive means to identify rotation compared to previous methods. Using a single photon counter, variations in intensity of the scattered red light from one F_1-bound nanorod allowed precise measurement of changes in angular position of the rod below the diffraction limit of light, and also provided the ability to resolve the power stroke of the motor with a time resolution of 2.5 μs.

In the work reported here, the gold nanorod assay with a polarizing filter was used to assess the fraction of *E. coli* F_1-ATPase molecules observed to rotate. A digital color camera with a refresh rate of 50 fps was used to record fields of view of F_1-dependent rotation of nanorods that were determined to be rotating based on their ability to blink red and green. The fraction of rotating molecules observed under these conditions was found to be 5 to 30-fold higher than reported previously with other methods, such that the majority of molecules can be observed to rotate. These data indicate that rotational measurements made using gold nanorods provide information of the F_1-ATPase mechanism that is representative of the characteristics of the enzyme population as a whole.

Materials and methods

E. coli XL-10 strain (Greene and Frasch 2003), containing a His6 tag on the N-terminus of the α-subunit, and a gS193C mutation to provide a biotinylation site, was used to isolate F_1. Cells were grown as explained previously (Lowry and Frasch 2005). Membranes were obtained as in (Ishmukhametov et al. 2005), and were resuspended in Release buffer containing 5.0 mM TES, pH 7, 40 mM ε-amino-caproic acid, 1 mM EDTA, 1 mM DTT, 5.0% (v/v) glycerol. The mixture was centrifuged at 180,000×g for 1 h at 4 °C. The supernatant was mixed with 10-× Binding buffer (0.5M TRIS/pH 8.0, 1M KCl, 300 mM imidazole, 50 mM $MgCl_2$) at a ratio of 10:1 (v/v). Glycerol was then added to 15% (v/v). This mixture was then loaded onto a Ni-NTA column (0.8 cm in diameter, 1.5 cm^3 of resin), which was prepared by first washing with water, and then equilibrating the column with Wash buffer (50 mM TRIS/pH 8.0, 100 mM KCl, 30 mM imidazole, 5 mM $MgCl_2$, 15% glycerol). After binding the protein to the column, it was washed with 4 column volumes of Wash buffer. To elute F_1, 3 ml of Wash buffer, supplemented with 150 mM imidazole, was passed through the column.

In the process of biotinylating the purified F_1 enzyme, 200 μl of F_1 solution was mixed with equimolar amount of biotin-maleimide, and passed through a desalting column equilibrated with Wash buffer. Biotinylated F_1 was stored at 0.1 mg/ml at −80 °C until use. The protein preparation demonstrated high rotational activity for half a year.

Gold nanorods for rotational microscope assay were prepared as in Spetzler et al. (2006). It was found that F_1 binds to the surface of regular glass as sufficiently as it does to a Ni-NTA covered glass surface. A 5 μl droplet of biotinylated F_1 was incubated on the slide for 5 min then washed with F_1 buffer (50 mM Tris-Cl, pH 8.0, and 10 mM KCl). Nanodevice assembly was completed by addition of saturating amounts of avidin-coated nanorods and incubated for 5 min at room temperature. This allowed the avidin-coated gold nanorods to be captured by the biotinylated γ-subunit. The slide was washed thoroughly in F_1 buffer to remove excess nanorods and minimize nonspecific binding. Samples that were examined for rotation included a final buffer containing Mg^{2+}-NTP at a concentration that maximized F_1-ATPase activity.

The bulk activity of F_1 was measured in a buffer containing 20 mM KCl, 100 mM TRIS, pH 8.0, 1 mM $MgCl_2$, and 2 mM ATP or GTP at 25 °C. Activity was monitored with the coupled assay as described by Greene and Frasch (2003). The turnover for ATP and GTP hydrolysis was 108 and 130 s^{-1}, respectively.

Results

Figure 1 shows the amount of gold particles bound to the surface in a typical field of view under the microscope in the absence and presence of immobilized F_1. The F_1-ATPase was immobilized on the slide by incubating for

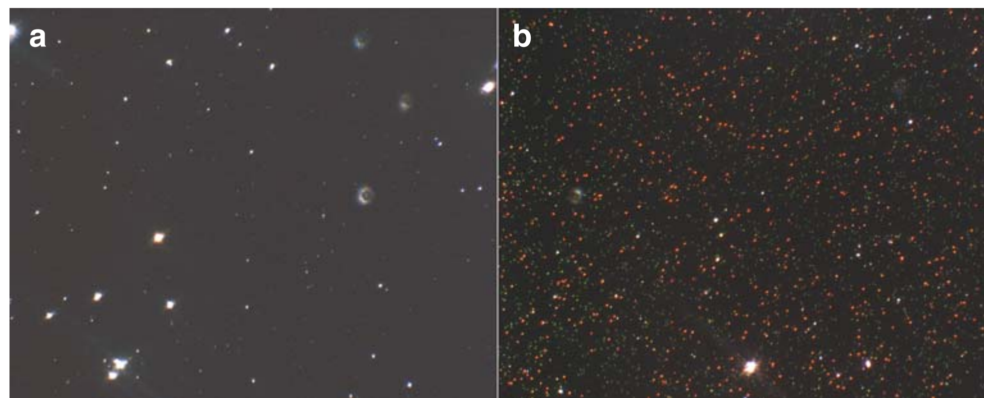

Fig. 1 A field of view under the microscope of gold particles bound to the slide in the absence (**a**) and presence (**b**) of 100 μg/ml F_1 immobilized to the surface. F_1 was immobilized to the surface prior to the addition of the gold nanoparticles. *White spots* are aberrations in the glass

5 min with 100 μg/ml of protein. Under these conditions, the average distance between immobilized F_1 molecules was 1±0.5 μm as measured by atomic force microscopy (Fig. 2), which is large enough to ensure that the gold rods are rarely bound to multiple F_1 molecules. The percent of nonspecifically bound nanorods is <0.1% of that bound when F_1 is present.

The method to prepare the gold nanorods used here is a two-step process that involves the initial formation of gold nanospheres that are subsequently elongated to nanorods with a yield of about 30% (Spetzler et al. 2006). Nanorods prepared in this manner provide a more sensitive measure of rotation compared to preparations that yield nearly 100% nanorods (Jana et al. 2001) due to the larger dynamic range of scattered light as a function of angular position to the polarizer. Consequently, the red spots in Fig. 1b are necessarily the result of light scattered from the long axis of bound nanorods, while the green spots are primarily the result of nanospheres that will not exhibit a rotational-dependent color change when viewed through a polarizer. Thus, for this study, green spots were not counted and not included in the calculation of the fraction of F_1 molecules observed to rotate.

Figure 3 shows a field of view under the microscope which is the first frame of a 500 frame movie of GTP-dependent rotation, where each frame had an exposure time of 20 ms. Determination that a nanorod was rotating was based on the fluctuation in the color between red and green during the course of each movie. Example movies are available as supplemental data. Yellow and red X's denote rods observed to rotate and not rotate, respectively. Figure 4 shows the first frame of a similar movie in the absence of nucleotide. Without substrate, a small percentage of the nanorods show some color fluctuation due to Brownian motion. While nucleotide-driven rotation can last for hours at a time, Brownian fluctuations are typically sporadic and do not show consistent rotation. The number of nanorods observed to rotate in the presence of ATP was also measured.

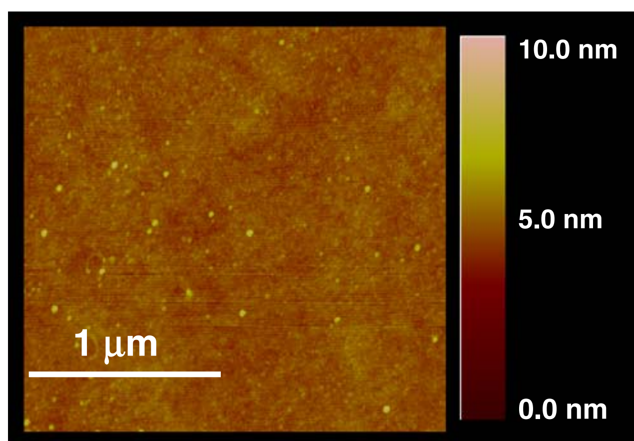

Fig. 2 Atomic force microscopy surface scan of F_1 bound to the surface of the slide after incubation of 100 μg/ml F1 followed by a wash

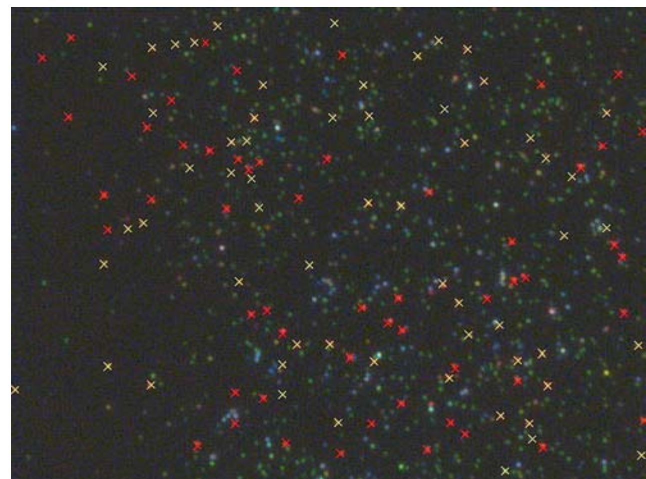

Fig. 3 The first frame from a movie depicting a microscope field of view of GTP-driven F_1 rotation. *Yellow* and *red Xs* denote rotating and non-rotating molecules, respectively. The GTPase activity of F_1 as measured with a coupled assay in the presence of 1 mM Mg^{2+}GTP was 130 s^{-1}. The movie can be viewed online in the supplemental information

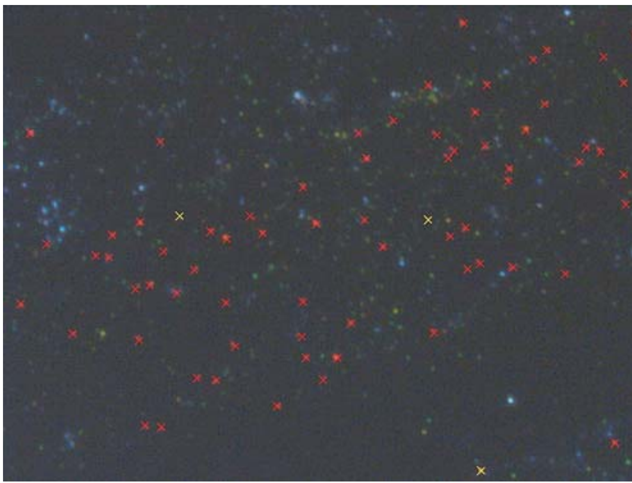

Fig. 4 The first frame from a movie depicting a microscope field of view of F_1 bound nanorods in the absence of nucleotides. *Yellow* and *red Xs* denote fluctuating and non-motile nanorods, respectively. The movie can be viewed online in the supplemental information

The fraction of nanorods observed to rotate was calculated by quantitating the number of rotating rods in each of several movies under the same conditions, then dividing by the total number of nanorods in those movies. As summarized in Fig. 5, the average number of F_1 molecules observed to rotate in the presence of GTP, ATP, and without nucleotide was ~50, ~25, and ~4% respectively. In some experiments, the fraction of molecules observed to rotate in the presence of GTP was as high as 65%.

Discussion

The results here clearly show that a majority of F_1 molecules can be observed to rotate with GTP. These data indicate that rotational measurements made using gold nanorods provide information about the F_1-ATPase mechanism that is representative of the characteristics of the enzyme population as a whole. It has been shown that *E. coli* F_1-ATPase is highly susceptible to inhibition that results from the entrapment of Mg^{2+}-ADP at a catalytic site (Hyndman et al. 1994; Bowler et al. 2006; Galkin et al. 2006). However, Mg^{2+}-GDP has been shown not to cause such an inhibition (Vasilyeva et al. 1980). This is the likely cause of the two-fold difference reported here in the abundance of rotating molecules powered by ATP and GTP, respectively.

The small fraction of molecules observed to rotate as measured by other approaches may be due to inherent limitations of those methods. When 40 nm gold nanospheres are used as the reporter group for rotation, they appear as light diffraction-limited spots ~250 nm in diameter. The eccentricity of the γ-subunit allows for about 5 nm of displacement during rotation (Oster et al. 2000; Sun et al. 2004). Thus, any bead that is attached near the center of the axis of rotation will show little variation in the location of the centroid of the spot when it rotates. Also, due to the substantial amount of surface variation on standard slides, it is possible for F_1 to become immobilized to the surface at an angle to the observation plane that virtually eliminates any variation in the location of the centroid.

The fraction of F_1 observed to rotate in single molecule measurements are likely an underestimate of the total number of those rotating on the slide due to several factors. First, it is possible that surface interactions prohibit rotation. The surface of the slide is not flat relative to the size of the F_1-ATPase and the detection probes. Consequently, it is likely that a fraction of the F_1 molecules bind to the surface at an angle that causes the probe to come in contact with the surface at some point in its trajectory in a manner that interferes with rotation.

Second, Spetzler et al. (2006) have shown that data acquisition rates of at least 50 kHz are required to resolve the 120° rotational steps (0.24 ms duration) of the *E. coli* F_1 γ-subunit that occur between the relatively long 8 ms pauses in rotation. Consequently, in the work presented here, rotation is evident in the movies acquired with a 50 fps color camera due to a strobe effect between rotation rate and the refresh rate of the camera. The strobe effect may not be apparent if the dwell positions for a given functioning F_1 motor are oriented in the field of view relative to the plane of polarization in such a way that there is very little dynamic range in the light intensity scattered by the nanorod. When this occurs, the apparent color of the nanorod will not fluctuate between red and green as a function of time.

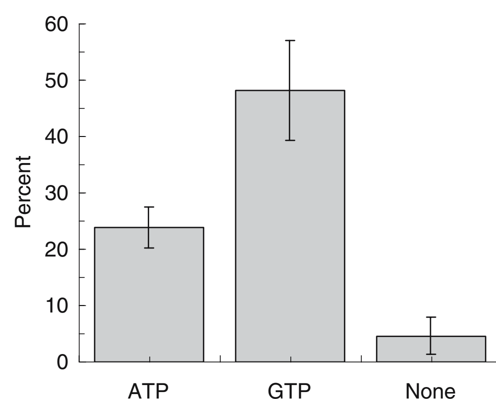

Fig. 5 Percent of nanorods directly observed to fluctuate between *red* and *green* in the presence of ATP, GTP, or in the absence of a nucleotide. Data were compiled from 11, 6, and 9 movies for GTP, ATP, and without nucleotide, respectively. Example movies of each condition can be viewed online in the supplemental information

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