Rotary torque produced by proton motive force in FoF₁ motor

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Abstract

We have attempted direct observation of the light-driven rotation of a FoF₁-ATP motor. The FoF₁-ATP motor was co-reconstituted by the deletion-δ subunit of FoF₁-ATP synthase with bacteriorhodopsins (BRs) into a liposome. The BR converts radiation energy into electrochemical gradient of proton to drive the FoF₁-ATP motor. Therefore, the light-driven rotation of FoF₁-ATP motor has been directly observed by a fluorescence microscopy using a fluorescent actin filament connected to β-subunit as a marker of its orientation. The rotational torque value of the Fo motor was calculated as 27.93 ± 1.88 pN nm. The ATP motor is expected to be a promising rotary molecular motor in the development of nanodevices.

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and β3δ) connecting Fo to F1. The δ-subunit is attached to an α-subunit in F1 and another segment connected to the β3 subunit of Fo with the β2 co-consisting of a ‘stalk’ in the peripheral FoF1-ATPase. If the δ-subunit is deleted, only one center ‘stalk’ (ε, γ) is present in the FoF1-ATP motor that is suitable for the development of nanotechnology to design and produce a new nanomotor. For this purpose, in the previous paper [8], we had developed a new model using hybrid δ-free FoF1-ATPase of chromatophore, to image the clockwise rotation of the ATP motor. Here, we further developed this new vivid model using the FoF1-ATP synthase and bacteriorhodopsins (BRs) co-reconstituted into a liposome. An artificial δ-free FoF1-ATP motor was constructed with ε, β3, ε, γ, and cα subunits as rotator and a, b2 as stator. The BR converts radiation energy into electrochemical gradient of proton to drive the FoF1-ATP motor. Using a fluorescent actin filament attached to the β-subunit, the light-driven rotation of FoF1-ATP motor was observed directly, viewed from Fo to F1 side under a fluorescent microscope.

Materials and methods

Reagents. Streptavidin was purchased from Sigma. Maleimido-C3-NTA and Biotin-AC5 Sulfo-OSu were purchased from Dojindo (Japan). 1,2-Dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-biotinyl was from Avanti. The purified bacteriorhodopsin was kindly provided by Dr. Zhong Sheng (Institute of Biophysics, Chinese Academy of Sciences).

Preparation of fluorescent actin filaments. The G-actin was co-labeled with FITC and Maleimido-C3-NTA−Ni2+ in buffer (50 mM Hepes–KOH (pH 7.6), 4 mM MgCl2, and 0.2 mM ATP) overnight at 4 °C. The free FITC and Maleimido-C3-NTA (Ni−NTA) were removed by a desalting column. Then the labeled G-actin was polymerized in buffer containing 50 mM/L Hepes–KOH (pH 7.6), 50 mM/L KC1, 4 mM/L MgCl2, and 2 mM/L ATP.

Preparation for immobilization of proteoliposomes in the experimental system for observation. Biotin-AC5-Sulfo-OSu was linked to the polylsine which had previously coated on the bottom of the dishes, then 100 μL of 10 mM streptavidin was added onto the dish bottom. After 5 min, the free streptavidin was washed. The proteoliposomes were conjugated by lipid–biotin-streptavidin–biotin–polylysine to the glass surface. And then the FITC-labeled F-actin filaments were attached to the β-subunit of F1 part through the His-tag with Maleimido-C3-NTA, as a marker of orientation for observation under an Olympus IX71 fluorescent microscope equipped with an ICCD camera.

Purification of the β-subunit, F1 (ε3β3ε10his-tag3γ3), and FoF1-ATPase. The F1-ATPase coding sequence was isolated from thermophilic bacterium PS3, and site-directed mutations of a cys193ser and gama107cys were introduced and a 10 histidine tag was inserted downstream of the initiation codon, the mutated construct, pGEM-MH, was then cloned into the expression plasmid pQE-30, and the expression plasmid pQE-MH was inserted into Escherichia coli JM103(uncB-UncD) in which a majority of F1-ATPase genes have been eliminated. Thermophilic bacterium, Bacillus PS3β10his-tag subunit (TFβ3) and F1-ATPase (ε3β3ε10his-tag3γ3) were expressed and purified as in [9], in which the JM103 strain expressing F1-ATPase was cultured in 2x YT medium (AMP−) for 3–4 h at 37 °C. When the A660 was increased to 0.6–0.8, the expression of the F1-ATPase was induced by addition of 1 mmol/L isopropylthio-β-n-galactoside for 3 h. Cells were harvested by centrifuging for 15 min at 4000g and cell extracts were prepared using lysozyme (1 mg/mL/sonication (5 min) in 50 mL/M Tris–HCl (pH 8.0) buffer containing 0.5 mol/L NaCl and 1 mmol/L phenylmethylsulfonyl fluoride. The extracts were incubated at 60 °C for 30 min, and TFβ3 was purified using Ni2+-NTA affinity chromatography at 4 °C. FoF1-ATPase (ε3β3ε10his-tag3γ3) was purified as described in [10] at 25 °C. The FoF1-ATP synthase also from the E. coli JM103(uncB-UncD) was purified as described by Foster [9]. The mutant ATP synthase containing the His-tag could be isolated with Ni−NTA column. FoF1-ATPase was eluted with buffer B containing 0.05% lysolecithin and 250 mM imidazole at 4 °C, and then further purified by a gel filtration column (Superdex 200 HR 10/30 Pharmacia). The purified protein was analyzed by SDS-PAGE.

Results and discussion

FoF1-ATPase from the E. coli JM103(uncB-UncD) was purified as [9]. The purified BR and FoF1-ATPase were co-reconstituted into a liposome. The liposome was prepared by reverse-phase evaporation with the mixture of soybean lipid and 1,2-dipalmitoyl-sn-glycero-3 phosphoethanolamine-N-biotinyl (molar ratio: 7:0.001) [11]. The molar ratio of BR and FoF1-ATPase was about 100:1, and that of lipids and protein was 30:1 (w/w). This is able to gain about one FoF1-ATPase and more than 20 BR molecules in one proteoliposome.

The procedure of the deletion δ-subunit of FoF1-ATPase is briefly shown in Figs. 1A–D. The proteoliposome containing FoF1-ATPase and BR was incubated in the buffer with 2 M LiCl, 0.1 mM Tricine−NaOH, 10 mM MgCl2, and 1 mM ATP for 20 min at 4 °C. Then the proteoliposome was washed with the buffer and isolated by centrifuging. As the treatment of FoF1-ATPase with LiCl removed not only β subunits but also some α and δ subunits, the LiCl-treated proteoliposome was co-incubated with purified ε3β3ε10his-tag3γ3 subunit at 4 °C for 60 min (for each divided subunit of F1-ATP with buffer 0.1 mM Tricine−NaOH, 10 mM MgCl2, 50 mM KCl, 0.5 mol/L NaCl, and 1 mM ATP), and then further incubated 37 °C for 60 min (for reconstructed δ-free FoF1-ATPase). The proteoliposome was further isolated to obtain the reconstituted δ-free FoF1-ATPase.

The ATP hydrolysis/synthesis activity assay was used to evaluate the reconstituted FoF1-ATPase [12]. As shown in Table 1, before the LiCl treatment, FoF1-ATPase in the proteoliposome had a good ATP hydrolysis/synthesis activity. Most of ATP hydrolysis/synthesis activity was lost in the LiCl-treated FoF1-ATP synthase. After incubation with F1(ε3β3ε10his-tag3γ3), 53% of its hydrolysis activity was restored. However, the ATP synthesis activity of the δ-less proteoliposome was only restored to about 6%, which was much lower than that of the ATP hydrolysis activity (53%). If only the purified β-subunit was used instead of F1(ε3β3ε10his-tag3γ3), the ATP hydrolysis activity was restored by about 5%. These
results indicated that \( \beta \) subunits were removed from FoF1-ATPase together with some \( \alpha \) and \( \delta \) subunits with the LiCl-treatment. After the incubation of \( F_1(\alpha_3\beta_10\cdot\text{his-tag})_3 \) with the LiCl-treatment of FoF1-ATPase, some of FoF1-ATPase were reconstituted with \( \beta \)- and \( \alpha \)-subunits to be only \( \delta \)-free. The FoF1-ATPase was used as the new ATP motor model for the clockwise rotation. The FoF1-ATPase could be stored at \(-70^\circ\text{C}\) with 20\% glycerol for 3 months before using.

The imaging of the FoF1-ATP motor rotation is shown in Fig. 2. A FITC-labeled actin filament was attached to the \( \beta \)-his-tag subunit in the proteoliposome, which was immobilized onto the glass surface through the biotin–streptavidin–biotin coupling. The sample was exposed
under the cooled light source with a 570 nm filter for 30 min to initiate the rotation of the FoF1-ATP motor. Before illumination, the buffer containing 2 mM NaN3 and 2 mM ATP was infused into the chamber, the clock-wise rotation of actin filament was observed directly in an Olympus IX71 fluorescent microscope equipped with an ICCD camera (Roper Scientific, Pentamax EEV 512 × 512 FT). Fig. 3 shows an example of the sequential images of a rotary fluorescent actin filament attached to FoF1-ATP motor with 100 ms time interval between each image. It is clear that the rotational orientation is clock-wise viewed from the Fo side to F1. If NaN3/ATP were not used in the buffer, no rotary filament was found. The addition of NaN3/ATP to the proteoliposomes not only inhibited the hydrolysis activity of the FoF1-ATPase, but also helped to hold force between the β3 and γ to tightly band them together [13]. Therefore, the rotary fluorescent actin filament attached to the β-subunit of F1 was clockwise observed directly from the Fo side to F1.

Worthy of mention is that in our experiment, the rotation event of FoF1-ATP motor was rare. On an average about 1/200 filaments started to rotate after illumination while others were immobile on the glass surface. Although the proteoliposome was fixed to the glass surface through the lipid–biotin–streptavidin–biotin–polylysine coating bottom of the dishes, some of the filaments were still torn off near the attachment point and floated into solution (about 1/300). We have found more than 20 rotary filaments connected to FoF1-ATP motor and the rotation of six filaments with different lengths is shown in Fig. 4. It can be seen that the rotary rates of many filaments were not constant with some of them having occasional pause and imaged even backwards. This phenomenon may be caused by the frictional role of the protein same as that in F1-motor imaging [14].

Several control experiments were performed to confirm that the rotation of the ATP motor is regulated by electrochemical gradient of proton. As shown in Fig. 5, Fig. 5A is the rotating filament stopped immediately when 5 μl of 10 μM CCCP was added (arrowhead the curve). It was also found that if the sample is in dark or incubation with DCCD before the illumination, no rotary filament is observed (data are not shown). The reason is that CCCP destroyed the proton driven force and DCCD was an inhibitor of Fo channel. These results demonstrated that the rotation of filaments depended on the proton driven force produced in Fo. It is interesting that the proteoliposomes can be keeping up the electrochemical gradient of protons for a long time. After the illumination, some filaments rotated continuously for more than 20 min. This phenomenon could be explain as the following two reasons: First, in our experiment, many proteoliposomes consisted of one FoF1 motor and a lot of BR molecules converting radiation energy to generate protons; Second, the proteoliposome seems to be a reservoir to store the energy that was converted by BR and the proton-driven motor rotary in Fo c-ring was like a strobe of the reservoir to
release protons, and also coupled a stalk ($e, \gamma$) of F1 to carry out rotation of the $\alpha_3\beta_3$ hexamer with high load, but not ATP synthesis.

We further characterized the rotation speed of the ATP motor. As shown in Fig. 6, the rotation speed decreased with the length of filaments ranging from 1.7 to 3.3 $\mu$m. This indicated that the rotation speed of motor depended on the F1 load. The frictional torque of the rotation is calculated by the equation developed by: 
\[ \tau = \frac{4n\pi^2r^3}{3\ln(L/2r) - 0.447} \]
where $n$ is the rotary rate, $\eta (10^{-3} N\cdot m/s^{2})$ is the viscosity of the medium, $L$ is the length of actin filament, and $r$ ($5 \text{ nm}$) is the radius of filament [14]. The averaged frictional torque calculated from the six actin filaments in Fig. 6 is 27.93 ± 1.88 pN nm. It has been reported that the rotary torque stalls a load of about 40 pN in the ATP hydrolysis driven in F1, and about 24 pN of proton driven in Fo [15,16]. The rotary torque produced in our experiment was closed to this one.

A new $\delta$-free FoF1-ATP motor was constructed in the proteoliposome to provide a feasible model to study the clockwise rotation of the ATP motor. Using this model, we have directly observed the rotary torque produced by Fo c-ring rotation of a FoF1 motor. The light-driven rotation of the motor has been successfully observed for a certain period of time. The further optimization and development of the proteoliposomes system is underway to make it an important model for the investigation of the coupling of Fo to F1-ATP motor and a useful biomolecular motor in nanotechnology.

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