Direct Observation of the Rotation of \( \epsilon \) Subunit in F\(_1\)-ATPase*

(Received for publication, April 27, 1998, and in revised form, June 4, 1998)

Yasuuki Kato-Yamada§§, Hiroyuki Nogi§, Ryohei Yasuda§§, Kazuhiro Kinosita Jr.§§, and Masasuke Yoshida§§

*From the 3Research Laboratory of Resources Utilization, Tokyo Institute of Technology, 4259 Nagatsuta, Yokohama, 226-8503, Japan, the 3Department of Physics, Faculty of Science and Technology, Keio University, 3-14-1, Hiyoshi, Yokohama 223-8522, Japan, and 3CREST (Core Research for Evolutional Science and Technology) Genetic Programming Team 13, Teikyo University Biotechnology Research Center 3F, 907 Nagowa, Miyamae, Kawasaki 216-0011, Japan

Rotation of the \( \epsilon \) subunit in F\(_1\)-ATPase from thermophilic Bacillus strain PS3 (TF\(_1\)) was observed under a fluorescence microscope by the method used for observation of the \( \gamma \) subunit rotation (Nogi, H., Yasuda, R., Yoshida, M., and Kinosita, K., Jr. (1997) Nature 386, 299–302). The \( \alpha_3\beta_3\gamma_3\epsilon \) complex of TF\(_1\) was fixed to a solid surface, and fluorescently labeled actin filament was attached to the \( \epsilon \) subunit through biotin-streptavidin. In the presence of ATP, the filament attached to the \( \epsilon \) subunit rotated in a unidirectional manner. The direction of the rotation was the same as that observed for the \( \gamma \) subunit. The rotational velocity was slightly slower than the filament attached to the \( \gamma \) subunit, probably due to the experimental setup used. Thus, as suggested from biochemical studies (Aggeler, R., Ogilvie, L., and Capaldi, R. A. (1997) J. Biol. Chem. 272, 19621–19624), the \( \epsilon \) subunit rotates with the \( \gamma \) subunit in F\(_1\)-ATPase during catalysis.

\( \alpha \), \( \beta \), and \( \gamma \) subunits of the F\(_1\)-ATPase catalyze ATP synthesis coupled with the proton flow across the energy-transducing membranes such as the plasma membrane of bacteria, mitochondrial inner membrane, and thylakoid membrane of chloroplast (1–4). F\(_1\)-ATPase is the water-soluble portion of F\(_0\)F\(_1\)-ATP synthase and contains a catalytic core for ATP synthesis and ATP hydrolysis. The F\(_1\)-ATPase consists of five kinds of subunits with a stoichiometry of \( \alpha_3\beta_3\gamma_1\delta_1\epsilon_1 \). The catalytic sites of ATP synthesis and ATP hydrolysis are located mainly on the \( \beta \) subunits, and noncatalytic nucleotide binding sites are located mainly on the \( \alpha \) subunit (5). The \( \alpha_3\beta_3\gamma \) subcomplex of F\(_1\)-ATPase is regarded as a minimum stable complex which has catalytic features similar to F\(_0\)-ATPase (6–8). Three catalytic sites of F\(_\gamma\)-ATPase exhibit strong negative cooperativity in ATP binding and positive cooperativity in ATP hydrolysis. To explain these characteristics, a binding change mechanism was proposed (3, 4) and has been widely accepted. In the binding change mechanism, all three \( \beta \) subunits in F\(_2\)F\(_1\)-ATP synthase are in different states at a given moment and alternately exchange their states during ATP synthesis and hydrolysis. The physical rotation of the \( \gamma \) subunit within the \( \alpha_3\beta_3\gamma \) hexamer was hypothesized as a mechanism for the binding change to occur (3), and a crystal structure of bovine mitochondrial F\(_1\)-ATPase in which a cylinder of the \( \alpha_3\beta_3\gamma \) hexamer is penetrated by the coiled-coil structure of the \( \gamma \) subunit gave the hypothesis more reality (5). Biochemical (9, 10) and optical (11) analyses provided support for the rotation of the \( \gamma \) subunit, and finally, the rotation was directly observed as the rotation of a fluorescently labeled actin filament attached to the \( \gamma \) subunit (12). Driven by ATP hydrolysis, the \( \gamma \) subunit rotated for several minutes in the direction predicted from the crystal structure of bovine mitochondrial F\(_1\). To obtain further insight into the mechanism of this enzyme, it is necessary to identify each subunit of F\(_2\)F\(_1\)-ATP synthase as either a rotor or stator subunit.

The \( \epsilon \) subunit, the smallest subunit of bacterial and chloroplast F\(_1\)-ATPases, is an endogenous ATPase inhibitor (14–16). According to recent structural analyses, the \( \epsilon \) subunit of Escherichia coli F\(_1\)-ATPase consists of an N-terminal \( \beta \)-sandwich and a C-terminal \( \alpha \)-helical domain (17, 18). The \( \epsilon \) subunit interacts with the \( \gamma \) subunit (19) and the analysis of a chimera complex from a thermophilic Bacillus PS3 (TF\(_1\)) and chloroplast F\(_1\)-ATPase indicated that the \( \epsilon \) subunit affects the ATPase activity of F\(_2\)-ATPase through the \( \gamma \) subunit (20). The subunit interface between the \( \gamma \) and \( \epsilon \) subunits has been explored by the cross-linking and chemical modification (21, 22), and recent work by Aggeler et al. (23) suggested that the \( \epsilon \) subunit rotates together with the \( \gamma \) subunit. Previously we reported that the inhibitory effect of the \( \epsilon \) subunit on ATPase activity of TF\(_1\) was observed only at low concentrations of ATP. Unlike the case of E. coli F\(_1\)-ATPase where the \( \epsilon \) subunit tends to dissociate from F\(_0\)-ATPase during multiple turnovers of ATPase reaction, the \( \epsilon \) subunit of TF\(_1\) remains associated with the \( \alpha_3\beta_3\gamma \) portion during catalysis (24). Taking advantage of this stable association of the \( \epsilon \) subunit, we observed directly the rotation of the \( \epsilon \) subunit in TF\(_1\).

EXPERIMENTAL PROCEDURES

Preparation of the Mutant \( \alpha_3\beta_3\gamma \) Complex and \( \epsilon \) Subunit of TF\(_1\)—Wild-type \( \epsilon \) subunit of TF\(_1\) does not contain cysteine. To ensure specific modification, a mutant \( \epsilon \) subunit (H38C) of TF\(_1\) was generated by the method of Kunkel et al. (25). A primer oligonucleotide (5′-AAGCGGATGGCATCCGGGCAAAGTG-3′) which contained substitution corresponding to H38C mutation and a new EcoT22I site, 1 was used as a primer. F\(_1\)-ATPase from the thermophilic Bacillus PS3; MOPS, 3-(N-morpholino)propanesulfonic acid; \( \alpha_3\beta_3\gamma \), a mutant (\( \alpha \)=c193S, \( \gamma \)=S107C, H38C-residue tagged in N terminus of the \( \beta \) subunit) \( \alpha_3\beta_3\gamma \) complex of TF\(_1\), were biotinylated with biotinylated \( \gamma \)-Cys-107; \( \alpha_3\beta_3\gamma \), a complex of \( \alpha_3\beta_3\gamma \) and the wild-type \( \epsilon \) subunit; \( \alpha_3\beta_3\gamma \), a mutant (\( \alpha \)=c193S, \( \gamma \)=H38C, His107C-residue tagged in N terminus of the \( \beta \) subunit) \( \alpha_3\beta_3\gamma \) complex of TF\(_1\), with streptavidin bound to biotinylated \( \epsilon \)-Cys-38; SA, streptavidin; DTT, dithiothreitol; AMPPNP, adenosine 5′-[\( \gamma \)-32P]triphosphate; Ni-NTA, nickel-nitrilotriacetic acid.

This paper is available on line at http://www.jbc.org
was used to introduce mutation to the expression plasmid of TF, ε, pTE2 (24). The mutant ε subunit was expressed in E. coli BL21(DE3) and purified as described previously (20) except that all buffers contained 1 mM DTT. The expression plasmid for the cysteine-less, His-tagged mutant (αC193S, Hisγ-γtags in β N termini) αβγ complex was generated from the expression plasmid used for the observation of the rotation of the γ subunit (12). Its BglII-NheI fragment was exchanged by that of wild-type plasmid (pKABG1) (7), and the mutation γS107C was reverted to serine. The αβγ complex was purified as described previously (7).

Preparation of Streptavidin-attached αβγ Complex of TF—Purified αβγ ε subunit was incubated at 23°C with 2 mM DTT for 15 min and passed through a Sephadex G-25M column equilibrated with 50 mM Tris-HCl (pH 8.0) and 100 mM KC1. Then, 50 mM N'-[β-(N-maleimidomethyl)-ethyl]-N-piperazinyl D-biotinamide (Dojindo) dissolved in dimethyl sulfoxide was added to the ε subunit solution (100 μM) to give a final concentration of 1 mM and incubated for 2 h at 23°C. The reaction was quenched by the addition of 7 mM DTT. The biotinylated ε subunit was allowed to bind to the αβγ γ complex, and the αβγγ complex formed was purified as described previously (24). The αβγ γ complex obtained was then mixed with 10 molar excess of streptavidin (SA) and incubated for 20 min at 23°C. Excess streptavidin was removed by G4000SWXL (Tosoh) gel filtration high performance liquid chromatography, and the fraction containing the αβγγ complex (the superscript SA designates the subunit labeled with biotin-streptavidin complex) was concentrated by Microcon-100 (Amicon).

Observation of Rotation—The rotation of the ε and γ subunits was observed by the same experimental setup as that used for the rotation of the γ subunit in the previous report (12, 13, Fig. 1). The ATP concentration was fixed at 2 mM in an ATP regenerating system, containing 0.2 mg/ml creatine kinase and 2.5 mM creatine phosphate. Rotation was observed at 23°C on an inverted fluorescence microscope (IX70, Olympus), and images were recorded with an SIT camera (C2741-08, Hamamatsu Photonics) on an 8-mm video tape. The rotation angle of the filament was estimated from the circular movement of the phosphor image (as in Ref. 12) using a manual (26).

RESULTS AND DISCUSSION

Experimental Setup—Three kinds of subunit complexes were used for experiments; the (αC193S, εH38C, His-tag) αβγε complex for observing the rotation of the ε subunit, and the (αC193S, γS107C, His-tag) αβγγ complex and the (αC193S, His-tag) αβγγ complex were almost the same; 57 s for 2 mM ATP supplied, continuous rotation of the actin filament attached to αβγγ ε was observed (Fig. 2). Rotation was absolutely dependent on ATP hydrolysis. In the absence of ATP, or in the presence of 2 mM ATP + 10 mM NaN₃, which inhibits ATPase activity of TF, no continuous rotation was observed (data not shown). The number of the rotating actin filaments per total actin filaments was less than 1%, 4- to 5-fold lower than the number observed for actin filaments attached to αβγγ ε. If the connection between the γ and ε subunits is weak, a rotating actin filament would be detached in a short time from the complex by the hydrodynamic frictional load. However, because the duration of the rotation which sometimes continued more than 5 min was apparently similar to the case when an actin filament was attached to the γ subunit, the γ-ε intersubunit connection might be strong enough to bear the hydrodynamic friction on the actin filament. The rotation was anti-clockwise when viewed from the membrane side. This direction is the same as that observed for the rotation of an actin filament attached to the ε subunit (12). According to the crystal structure of the bovine mitochondrial F₁ (5), when the ε (and γ) subunit(s) rotates in this direction, one β subunit experiences the transition in the order expected in the ATP hydrolysis reaction, AMPPNP-bound form, ADP-bound form, and empty form.

In the plot of rotational rate versus filament length (Fig. 3),
the same angular velocity. The fact that rotational rates of the γ subunit in αβγεSA and in αβγeSA were the same with each other (Fig. 3) suggests that the presence of the ε subunit in the αβγεSA complex does not impede the rotation of the γ subunit. Therefore, the apparent difference in the rotational rates between the ε-attached filament and the γ-attached filament appears to be caused from an experimental artifact at present. If an actin filament can bind to the ε subunit at Cys-38 only with nonhorizontal, downward angle (when a complex is viewed as in Fig. 1B), increased hydrodynamic friction near the surface (32) might slow the rotation of the filament. Another possible cause is the biotin-streptavidin connection through the single bond between the ε subunit and the actin filament. In principle, a single bond allows free rotation around the bond axis, and the rotation of the ε subunit in αβγeSA may not be transmitted at 100% efficiency to the rotation of the actin filament, resulting in the apparent slow rotation. This could happen to the rotation of the γ subunit-attached filament, but fortunately it seems not.

In summary, we show here exclusive evidence that the ε subunit rotates in F1-ATPase relative to the αβγ hexagon ring during catalysis. Further identification of the rotor and the stator subunits is required to know the coupling mechanism of F0F1-ATP synthase.

REFERENCES