Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 31, Issue of July 31, pp. 19375–19377, 1998

Direct Observation of the Rotation of ϵ Subunit in F_1-ATPase*

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

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Rotation of the ϵ 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 γ subunit rotation (Noji, H., Yasuda, R., Yoshida, M., and Kinosita, K., Jr. (1997) *Nature* 386, 299–302). The $\alpha_3\beta_3\gamma\epsilon$ complex of TF_1 was fixed to a solid surface, and fluorescently labeled actin filament was attached to the ϵ subunit through biotin-streptavidin. In the presence of ATP, the filament attached to ϵ subunit rotated in a unidirection. The direction of the rotation was the same as that observed for the γ subunit. The rotational velocity was slightly slower than the filament attached to the γ subunit, probably due to the experimental setup used. Thus, as suggested from biochemical studies (Aggeler, R., Ogilvie, I., and Capaldi, R. A. (1997) *J. Biol. Chem.* 272, 19621–19624), the ϵ subunit rotates with the γ subunit in F_1-ATPase during catalysis.

F_0F_1-ATP synthase catalyzes 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_0F_1-ATP synthase and contains a catalytic core for ATP synthesis and 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 hydrolysis are located mainly on the β subunits, and noncatalytic nucleotide binding sites are located mainly on the α subunits (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_1-ATPase (6–8). Three catalytic sites of F_1-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 β subunits in F_0F_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 γ subunit within the $\alpha_3\beta_3$ 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 $\alpha_3\beta_3$ hexamer is penetrated by the coiled-coil structure of the γ subunit gave the hypothesis more reality (5). Biochemical (9, 10) and optical (11) analyses provided support for the rotation of the γ subunit, and finally, the rotation was directly observed as the rotation of a fluorescently labeled actin filament attached to the γ subunit (12). Driven by ATP hydrolysis, the γ 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_0F_1-ATP synthase as either a rotor or stator subunit.

The ϵ subunit, the smallest subunit of bacterial and chloroplast F_1-ATPases, is an endogenous ATPase inhibitor (14–16). According to recent structural analyses, the ϵ subunit of *Escherichia coli* F_1-ATPase consists of an N-terminal β-sandwich and a C-terminal α-helical domain (17, 18). The ϵ subunit interacts with the γ subunit (19) and the analysis of a chimeric complex from a thermophilic *Bacillus* PS3 (TF_1)[1] and chloroplast F_1-ATPase indicated that the ϵ subunit affects the ATPase activity of F_1-ATPase through the γ subunit (20). The subunit interface between the γ and ϵ subunits has been explored by the cross-linking and chemical modification (21, 22), and recent work by Aggeler *et al.* (23) suggested that the ϵ subunit rotates together with the γ subunit. Previously we reported that the inhibitory effect of the ϵ 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 ϵ subunit tends to dissociate from F_1-ATPase during multiple turnovers of ATPase reaction, the ϵ 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 ϵ subunit, we observed directly the rotation of the ϵ subunit in TF_1.

EXPERIMENTAL PROCEDURES

Preparation of the Mutant $\alpha_3\beta_3\gamma$ Complex and ϵ Subunit of TF_1—Wild-type ϵ subunit of TF_1 does not contain cysteine. To ensure specific modification, a mutant ϵ subunit (H38C) of TF_1 was generated by the method of Kunkel *et al*. (25). A primer oligonucleotide (5′-AAGCGGAATGCATCCCGGCAAAATG-3′) which contained substitution corresponding to H38C mutation and a new *EcoT22*I site,

* This work was supported in part by Grant 07458158 from the Ministry of Education, Science, and Culture of Japan and grants from CREST (Core Research for Evolutional Science and Technology). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists.

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[1] The abbreviations used are: TF_1, F_1-ATPase from the thermophilic *Bacillus* PS3; MOPS, 3-(*N*-morpholino)propanesulfonic acid; $\alpha_3\beta_3\gamma^{SA}$, a mutant (αC193S, γS107C, His_{10}-tag in N terminus of the β subunit) $\alpha_3\beta_3\gamma$ complex of TF_1 with streptavidin bound to biotinylated γ-Cys-107; $\alpha_3\beta_3\gamma^{SA}\epsilon$, a complex of $\alpha_3\beta_3\gamma^{SA}$ and the wild-type ϵ subunit; $\alpha_3\beta_3\gamma\epsilon^{SA}$, a mutant (αC193S, ϵH38C, His_{10}-tag in N terminus of the β subunit) $\alpha_3\beta_3\gamma\epsilon$ complex of TF_1 with streptavidin bound to biotinylated ϵ-Cys-38; SA, streptavidin; DTT, dithiothreitol; AMPPNP, adenosine 5′-(β,γ-imino)triphosphate; Ni-NTA, nickel-nitrilotriacetic acid.

was used to introduce mutation to the expression plasmid of $TF_1\epsilon$, 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, His_{10}-tags in β N termini) $\alpha_3\beta_3\gamma$ complex was generated from the expression plasmid used for the observation of the rotation of the γ subunit (12). Its *Bgl*II-*Nhe*I fragment was exchanged by that of wild-type plasmid (pKABG1) (7), and the mutation γS107C was reverted to serine. The $\alpha_3\beta_3\gamma$ complex was purified as described previously (7).

Preparation of Streptavidin-attached $\alpha_3\beta_3\gamma\epsilon$ Complex of TF_1—Purified (H38C) ϵ subunit was incubated at 23 °C with 2 mM DTT for 15 h and passed through a Sephadex G-25M column equilibrated with 50 mM Tris-HCl (pH 8.0) and 100 mM KCl. Then, 50 mM *N*′-[2-(*N*-maleimido)-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 $\alpha_3\beta_3\gamma$ complex, and the $\alpha_3\beta_3\gamma\epsilon$ complex formed was purified as described previously (24). The $\alpha_3\beta_3\gamma\epsilon$ 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 $\alpha_3\beta_3\gamma\epsilon^{SA}$ (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 centroid of the filament image calculated using a digital image processor (DIPS-C2000, Hamamatsu Photonics) (12, 13).

Other Materials and Procedures—For a control, $\alpha_3\beta_3\gamma^{SA}$ in which streptavidin was attached to the biotinylated γ-Cys-107 was prepared from the mutant (αC193S, γS107C, His_{10}-tags in β N termini) $\alpha_3\beta_3\gamma$ complex as described (12, 13). A $\alpha_3\beta_3\gamma^{SA}\epsilon$ complex was reconstituted from $\alpha_3\beta_3\gamma^{SA}$ and the wild-type ϵ subunit (24). The purity of the complexes was checked by 6% polyacrylamide gel electrophoresis without a denaturing reagent (24). ATPase activity was measured at 23° C in the presence of an ATP regenerating system in 10 mM MOPS-KOH (pH 7.0) buffer containing 50 mM KCl, 4 mM $MgCl_2$, 50 μg/ml pyruvate kinase, 50 μg/ml lactate dehydrogenase, 2.5 mM phospho*enol*pyruvate, 0.2 mM NADH, and 2 mM ATP. Steady-state ATPase activities of the (αC193S, His-tag) $\alpha_3\beta_3\gamma$ complex and the (αC193S, His-tag) $\alpha_3\beta_3\gamma\epsilon$ complex were almost the same; 57 s^{-1} and 58 s^{-1} (expressed as a turnover rate), respectively. Rabbit skeletal actin filaments were biotinylated and stained with phalloidin-tetramethylrhodamine B isothiocyanate conjugate (as in Ref. 12 but without cross-linking). Streptavidin was purchased from Sigma as lyophilized powder. Experimental procedures of recombinant DNA were performed as described in a manual (26). *E. coli* strain JM109 (27) was used for the preparation of plasmids, and the strain CJ236 (25) was used for generating uracil-containing single-stranded plasmids for site-directed mutagenesis. Protein concentrations were determined by the method of Bradford (28) using bovine serum albumin as a standard or from the UV absorbance using an absorbance 0.45 at 280 nm for 1 mg/ml of the subunit complexes of TF_1 (29). Polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate was performed as described by Laemmli (30).

RESULTS AND DISCUSSION

Experimental Setup—Three kinds of subunit complexes were used for experiments; the (αC193S, ϵH38C, His-tag) $\alpha_3\beta_3\gamma\epsilon$ complex for observing the rotation of the ϵ subunit, and the (αC193S, γS107C, His-tag) $\alpha_3\beta_3\gamma$ and the (αC193S, γS107C, His-tag) $\alpha_3\beta_3\gamma\epsilon$ complexes for observing the rotation of the γ subunit. Ten histidine tags at the N termini of the β subunits were used for the immobilization of the complex on a solid surface, and α-C193S was for elimination of unwanted cysteine residues to ensure the specificity of the biotinylation (among five kinds of subunits of the wild-type TF_1, only the α subunit contains a cysteine residue, α-Cys-193). In addition to these two mutations, we introduced a cysteine at the position of ϵ-His38 which is supposed to face the F_0 side (opposite to the N termini of α and β subunits) in the structure of F_1-ATPase (31). The biotinylation at ϵ-Cys-38 did not impair the ability of the ϵ subunit to associate with the $\alpha_3\beta_3\gamma$ complex to form the $\alpha_3\beta_3\gamma\epsilon$ complex. Polyacrylamide gel electrophoresis without a denaturing reagent, in which two ($\alpha_3\beta_3\gamma$ and $\alpha_3\beta_3\gamma\epsilon$) complexes were electrophoresed as separate bands (24), gave only a single band at a corresponding position, ensuring the homogeneity of each complex prepared. After streptavidin was bound to the introduced biotin, we fixed $\alpha_3\beta_3\gamma\epsilon^{SA}$ to the beads which adhered on the glass plate, and a fluorescently labeled, biotinylated actin filament was attached to the ϵ subunit through biotin-streptavidin-biotin (Fig. 1). Fixation and actin filament attachment to $\alpha_3\beta_3\gamma^{SA}$ and $\alpha_3\beta_3\gamma^{SA}\epsilon$ were carried out in the same way.

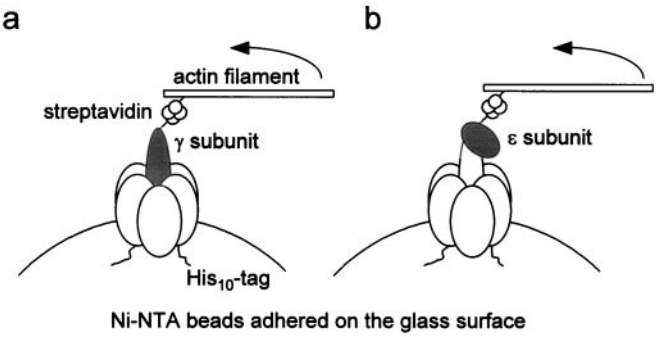

FIG. 1. **Schematic illustration of experimental setup for observation of the rotation.** *a*, rotation of the γ subunit (12, 13). The $\alpha_3\beta_3\gamma^{SA}$ complex was fixed through His-tags to the surface of the Ni-NTA beads (13) adhered on the bottom cover glass. The biotinylated γ-Cys-107 and a biotinylated actin filament, which was fluorescently labeled, were connected by streptavidin. The recorded images correspond to the view from the top in this figure (mirror image of the bottom view). The observed direction of the rotation was indicated by an *arrow*. The observation of the rotation of the γ subunit in $\alpha_3\beta_3\gamma^{SA}\epsilon$ was carried out in the same way. *b*, rotation of the ϵ subunit. An actin filament was attached to the ϵ subunit in $\alpha_3\beta_3\gamma\epsilon^{SA}$. Other details are the same as in *a*.

Characteristics of the Rotation—When 2 mM ATP was supplied, continuous rotation of the actin filament attached to $\alpha_3\beta_3\gamma\epsilon^{SA}$ 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 NaN_3 which inhibits ATPase activity of TF_1, 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 $\alpha_3\beta_3\gamma^{SA}$. 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 γ subunit (12). According to the crystal structure of the bovine mitochondrial F_1 (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),

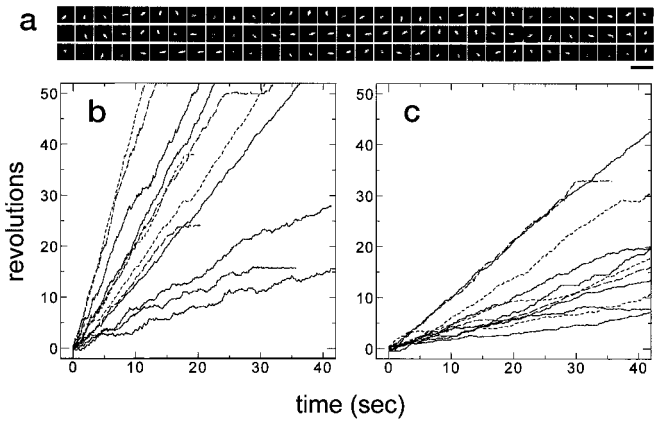

FIG. 2. **Rotation of the actin filament attached to the ϵ subunit in the presence of 2 mM ATP.** *a*, an example of sequential images of a rotating actin filament attached to the ϵ subunit in $\alpha_3\beta_3\gamma\epsilon^{SA}$. Length of the filament from rotational axis to the tip was 1.6 μm. Rotational rate was 1.0 rps. Time interval between images was 100 ms. The *scale bar* denotes 5 μm. *b*, examples of time course of the rotation of the actin filament. Length of the filaments presented here is 0.5–1.4 μm. Only the filaments that rotated around one end are shown. Each *line* represents one filament. *Solid lines* indicate the rotation of the ϵ subunit in $\alpha_3\beta_3\gamma\epsilon^{SA}$ while *dotted lines* and *broken lines* indicate the rotation of the γ subunit in $\alpha_3\beta_3\gamma^{SA}$ and $\alpha_3\beta_3\gamma^{SA}\epsilon$, respectively. *c*, same as *b* except that the length of the filaments is more than 1.5 μm. Details of the experiments are described under "Experimental Procedures."

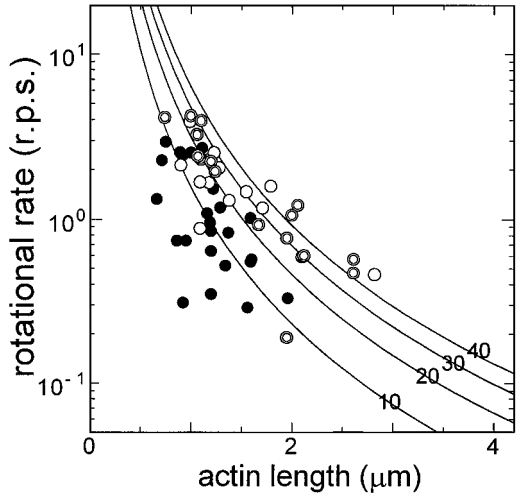

FIG. 3. **Rotational rate *versus* length of the actin filament.** Only the data for the filaments that rotated around one end are shown. Rotational rates were estimated by least square linear fitting on the time courses for more than 5 revolutions and expressed in rps. *Closed circles*, *double circles*, and *open circles* indicate results of $\alpha_3\beta_3\gamma\epsilon^{SA}$, $\alpha_3\beta_3\gamma^{SA}$, and $\alpha_3\beta_3\gamma^{SA}\epsilon$, respectively. *Solid lines* represent calculated rotational rate of the filaments with varying length which give a constant torque (hydrodynamic friction) value in pN·nm indicated on the lines. Hydrodynamic friction was calculated by $(4\pi/3)\omega\eta L^3/[\ln(L/2r) - 0.447]$, where ω is angular velocity, η (10^{-3} Nsm^{-2}) the viscosity of the medium, L the length of actin filament, and r (5 nm) the radius of the actin filament (12, 13, 32).

we notice the tendency that the rotation of the filament attached to $\alpha_3\beta_3\gamma\epsilon^{SA}$ was somewhat slower than that of the filament attached to $\alpha_3\beta_3\gamma^{SA}$ or to $\alpha_3\beta_3\gamma^{SA}\epsilon$. As a consequence, the apparent torque needed for rotation (12, 32) of the filament attached to $\alpha_3\beta_3\gamma\epsilon^{SA}$ at the observed rate was at most ~25 pN·nm, smaller than the corresponding values for $\alpha_3\beta_3\gamma^{SA}$ and $\alpha_3\beta_3\gamma^{SA}\epsilon$, ~40 pN·nm (Fig. 3). Because the cross-linking between the γ and ϵ subunits has little effect on ATPase activity (33–35), the γ and ϵ subunits are supposed to rotate together at the same angular velocity. The fact that rotational rates of the γ subunit in $\alpha_3\beta_3\gamma^{SA}\epsilon$ and in $\alpha_3\beta_3\gamma^{SA}$ were the same with each other (Fig. 3) suggests that the presence of the ϵ subunit in the $\alpha_3\beta_3\gamma\epsilon$ 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. 1*B*), 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 $\alpha_3\beta_3\gamma\epsilon^{SA}$ 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 F_1-ATPase relative to the $\alpha_3\beta_3$ hexagon ring during catalysis. Further identification of the rotor and the stator subunits is required to know the coupling mechanism of F_0F_1-ATP synthase.

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