The γ subunit in chloroplast F_1-ATPase can rotate in a unidirectional and counter-clockwise manner

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Abstract Rotation of the γ subunit in chloroplast F_1-ATPase (CF_1) was investigated by using a single molecule observation technique, which is developed by Noji et al. to observe the rotation of a central γ subunit portion in the $\alpha_3\beta_3\gamma$ sub-complex of F_1-ATPase from thermophilic *Bacillus* PS3 (TF_1) during ATP hydrolysis [Noji, H. et al. (1997) Nature 386, 299–302]. We used two cysteines of the γ subunit (Cys-199 and Cys-205) of CF_1-ATPase, which are involved in the regulation of this enzyme, to fix the fluorochrome-labeled actin filament. Then we successfully observed a unidirectional, counter-clockwise rotation of the actin filament with the fluorescent microscope indicating the rotation of the γ subunit in CF_1-ATPase. We conclude that the rotation of the γ subunit in the F_1-motor is a ubiquitous phenomenon in all F_1-ATPases in prokaryotes as well as in eukaryotes.

Key words: F_1-ATPase; Rotation; γ Subunit; Chloroplast; Regulation

1. Introduction

F_oF_1-ATP synthase of bacteria, mitochondria and chloroplasts synthesizes ATP from ADP and P_i at the expense of a proton-motive force [1–3]. The enzyme consists of the membrane-embedded sector F_o responsible for proton translocation, and the extrinsic catalytic part F_1. The architecture of F_1 is very similar in various kinds of cells or organelles, respectively, and composed of five different subunits, $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$. Rotation of the central γ subunit in the $\alpha_3\beta_3$ headpiece of F_1 during the hydrolysis of ATP as suggested by Boyer [4], was experimentally supported by a biochemical approach [5], and by polarization anisotropy relaxation measurement of the fluorophore-labeled γ subunit of CF_1 [6]. Noji et al. directly observed the rotation of the γ subunit in an $\alpha_3\beta_3\gamma$ complex of TF_1 by means of a fluorochrome-labeled actin filament fixed to this subunit [7]. They reported a unidirectional, counter-clockwise, and continuous rotation driven by ATP hydrolysis. It suggests that the γ subunit may interact with three α/β couples sequentially step by step according to the catalytic reaction occurring at each of three catalytic sites. At very low concentration of ATP Yasuda et al. recently succeeded to observe this step-wise motion of the subunit with 120 degree steps [8], suggesting that the stepping observed by the actin filament directly reflects the catalysis occurring on each of three catalytic sites resides on γ subunits. Furthermore, Noji et al. [9] and Omote et al. [10] independently reported the rotation of the γ subunit in *E. coli* F_1-ATPase by using essentially the same kind of experimental approach.

On the other hand, Hartog and Berden [11] proposed a different catalytic mechanism which might exclude a simple rotation of the γ subunit in F_1-ATPase. Based on the results from energy transfer experiments between the γ subunit and catalytic sites on γ subunits [12,13] and on the ATPase activity of a complex formed from the isolated CF_1 $\alpha_3\beta_3$ subcomplex and recombinant γ subunit of which the C-terminus was partially deleted, Sokolov et al. recently proposed a conformation of the γ subunit of CF_1 in which the tip of the C-terminus of the γ subunit does not act as a spindle for rotation [14]. However, large differences in the conformations of the γ subunits of CF_1 and bacterial F_1 are not very likely as we succeeded to form an ATPase active chimeric complex with the α and β subunits from TF_1 and the recombinant γ subunit of CF_1 [15,16]. The resulting complex mostly maintained the features of TF_1 but was regulated like CF_1 by the formation and the reduction of a disulfide bridge located in this γ subunit.

To clarify whether the rotation of the γ subunit in F_1-ATPase is ubiquitous in nature, we here tried to demonstrate it in CF_1 by employing the method of Noji et al. [7]. As the actin filament was fixed to the γ subunit via a naturally occurring cysteine, the CF_1/actin derivative could be produced without any gene engineering technique. With this molecule we successfully observed a unidirectional, counter-clockwise rotation of the γ subunit in CF_1 with the fluorescent microscope.

2. Materials and methods

2.1. Chemicals

6-{*N*′-[2-(*N*-maleimide)ethyl]-*N*-piperazinylamido}hexyl D-biotinamide (biotin-$PEAC_5$-maleimide) was purchased from Dojindo laboratories (Japan). *N,N*-bis[2-hydroxyethyl]glycine (Bicine), 3-[*N*-morpholino]propanesulfonic acid (MOPS), *N*-ethyl-maleimide (NEM), dithiothreitol (DTT), and streptavidin were from Sigma (USA). All of other reagents were the highest grade commercially available.

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Abbreviations: Bicine, *N,N*-bis[2-hydroxyethyl]glycine; biotin-$PEAC_5$-maleimide, 6-{*N*′-[2-(*N*-maleimide)ethyl]-*N*-piperazinylamido}hexyl D-bioninamide; CF_1, chloroplast coupling factor 1; $CF_1(-\delta)$, δ-subunit deficient CF_1; DTT, dithiothreitol; EF_1, F_1-ATPase from *Escherichia coli* plasma membrane; TF_1, F_1-ATPase from thermophilic *Bacillus* PS3 plasma membrane; MK buffer, 10 mM MOPS-NaOH buffer (pH 7.0) and 50 mM KCl; NEM, *N*-ethyl-maleimide; MKB buffer, MK buffer plus 10 mg/ml bovine serum albumin; MKR buffer, MK buffer containing 50 mM Na_2SO_3, 6 mM glucose, 0.5% 2-mercaptoethanol, 30 U/ml catalase, and 0.4 mg/ml glucose oxidase; MOPS, 3-[*N*-morpholino]propanesulfonic acid; NEM, *N*-ethyl-maleimide

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2.2. Preparation of CF_1 from spinach

CF_1 was directly extracted with chloroform from thylakoid membranes prepared from market spinach [17] as previously described [18]. For the further purification, an Econo-Pac cartridge, *t*-butyl HIC (5 ml, Bio-Rad, USA) which was previously equilibrated with 50 mM Tris–HCl (pH 8.0), 1 mM ATP, 0.5 mM EDTA and 0.5 M $(NH_4)_2SO_4$ was used [19]. The crude extract dissolved in the same buffer was loaded on the column and the proteins were eluted from the column by the gradient of $(NH_4)_2SO_4$ from 0.5 M to 0 M. The major peak fraction eluted from the column was the δ-subunit deficient CF_1 ($CF_1(-\delta)$) (Fig. 1, lane 1). It was stored as ammonium sulfate precipitation at 4°C. Before use, the preparation was desalted by passage through a gel filtration column (TSK G-3000_{XL}, 14 mm × 300 mm, Tosoh, Japan) equilibrated with 10 mM MOPS-NaOH buffer (pH 7.0) and 50 mM KCl (MK buffer).

2.3. Biotin-streptavidin label of $CF_1(-\delta)$

The desalted $CF_1(-\delta)$ was first incubated with the equivalent molar ratio of NEM for 20 min at room temperature and unreacted NEM was removed by gel filtration chromatography as described above. The NEM-treated $CF_1(-\delta)$ was then reduced by the incubation with 20 mM DTT for 20 min at 37°C. After the removal of DTT in the solution by gel filtration with the same column, five-fold molar ratio of biotin-$PEAC_5$-maleimide was added to $CF_1(-\delta)$ solution and was incubated for 20 min at room temperature. Unreacted biotin-$PEAC_5$-maleimide was removed by gel filtration and streptavidin was attached to biotin on $CF_1(-\delta)$ by the addition of 10-fold molar excess streptavidin. Free streptavidin was then removed by a gel filtration column and the peak fraction containing streptavidin-labeled $CF_1(-\delta)$ was collected.

2.4. Measurement of the ATPase activity

The ATPase activity of $CF_1(-\delta)$ was measured according to the method described in [19]. The reaction mixture contained 4 mM ATP, 1 mM $MgCl_2$, 100 mM Na_2SO_3. The buffers used were 50 mM MOPS–KOH (pH 7.0), Tricine–KOH (pH 8.0), or Bicine–NaOH (pH 9.0).

2.5. Rotation assay

A flow chamber was constructed of two coverslips (bottom, 24 × 36 mm^2, top, 18 × 18 mm^2) separated by 50 μm spacers [7]. One chamber volume (about 10 μl) of 20 nM CF_1 solution was infused into the flow chamber and allowed to adhere to the glass surface for 2 min. The chamber was washed twice with three volumes of MK buffer plus 10 mg/ml bovine serum albumin (MKB buffer). Then, 160 nM fluorescently labeled actin filament in MKB buffer was infused and incubated for 15 min. After the washing with MKB buffer, the solution in the chamber was replaced with MK buffer containing 50 mM Na_2SO_3, 6 mM glucose, 0.5% 2-mercaptoethanol, 30 U/ml catalase, and 0.4 mg/ml glucose oxidase (MKR buffer) plus 20 mM ATP and 5 mM $MgCl_2$ to initiate ATP hydrolysis reaction. The chamber was observed on an inverted fluorescence microscope (IX70, Olympus, Japan). Fluorescence images were recorded with an intensified CCD camera (ICCD-350F, Video Scope, USA) on an 8 mm video tape and was analyzed by the image analysis software provided by Dr. Yasuda, R.

3. Results and discussion

3.1. Biotin label of CF_1-γ subunit

Spinach CF_1 has altogether 11 cysteines (α: 3 × 1; β: 3 × 1; γ: 4; ε: 1). All of these cysteines are potential candidates for the biotin-$PEAC_5$-maleimide labeling. However, the most reactive cysteine is Cys-322 on the γ subunit (see figure 1 of Ref. [6]). This cysteine is the penultimate amino acid from the C-terminus and should be located on the top of the molecule according to the MF_1 structure [20]. In order to get the filament fixed to the opposite pole of the γ subunit, we first treated $CF_1(-\delta)$ with NEM to block this cysteine. After the treatment, $CF_1(-\delta)$ was reduced by DTT. This reduction converts a disulfide group between Cys-199 and Cys-205 to the respective dithiol groups [21]. By the subsequent treatment with biotin-$PEAC_5$-maleimide the biotin label could be introduced at Cys-199 or Cys-205. Both positions are in the portion of the γ subunit that remained unresolved in the MF_1 structure [20] and located at the bottom pole of the F_1 molecule. Biotin labeled $CF_1(-\delta)$ was then analyzed by SDS-PAGE and the bands were visualized by streptavidin conjugated alkaline-phosphatase (Fig. 1, lane 3). Biotin was only incorporated into the γ and ε subunit. Kato et al. already reported that the ε subunit in TF_1 rotates together with the γ subunit [22]. Therefore we did not try to separate possible different CF_1/biotin derivatives from each other.

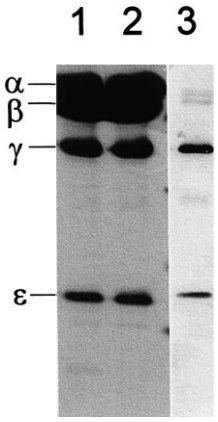

Fig. 1. Biotinylation of the γ subunit of CF_1. SDS-PAGE was performed on 15% (w/v) polyacrylamide gel. Samples were as follows: 20 μg of non-treated $CF_1(-\delta)$ (lane 1), 20 μg of biotinylated $CF_1(-\delta)$ (lane 2) and 3 μg of biotinylated $CF_1(-\delta)$ (lane 3). Proteins in lane 1 and 2 were stained with Coomassie brilliant blue R-250. Lane 3 was visualized by streptavidin-conjugated alkaline phosphatase.

3.2. ATPase activity of the modified CF_1

ATPase activity of streptavidin-labeled $CF_1(-\delta)$ was examined in the presence of 100 mM Na_2SO_3, which stimulates the ATPase activity of CF_1 [23], and compared with that of unlabeled $CF_1(-\delta)$. The ATPase activity of $CF_1(-\delta)$ labeled with streptavidin was 0.83 s^{-1} at 37°C, i.e. about 40% lower than that of unlabeled CF_1 but suitable for the rotation assay. The ATPase activity of the streptavidin-labeled $CF_1(-\delta)$ measured at 25°C (as for the rotation assay) was 1.31 s^{-1} at pH 8 and 1.97 s^{-1} at pH 9, i.e. six to seven-fold higher than that measured at pH 7 (0.21 s^{-1}). These rates are similar to those reported previously [24], but much slower than those obtained for the $\alpha_3\beta_3\gamma$ complex of TF_1 [7,8].

3.3. Observation of the rotation of CF_1-γ

The $CF_1(-\delta)$ derivative spontaneously fixed to the glass surface when it was infused into the flow chamber. When streptavidin-labeled $CF_1(-\delta)$ was employed and the fluorochrome labeled actin filament was infused, lots of actin filaments were observed under the fluorescence microscope but no filament was observed when biotin-labeled $CF_1(-\delta)$ was used instead of streptavidin-labeled $CF_1(-\delta)$, implying that the actin-filament was bound to $CF_1(-\delta)$ via streptavidin. Adhesion of $CF_1(-\delta)$ on the glass surface was disturbed when MKB buffer, which contains 10 mg/ml bovine serum albumin, was infused previously, and no binding of actin filaments was observed in this case.

As shown in Figs. 2 and 3, we could find a few filaments

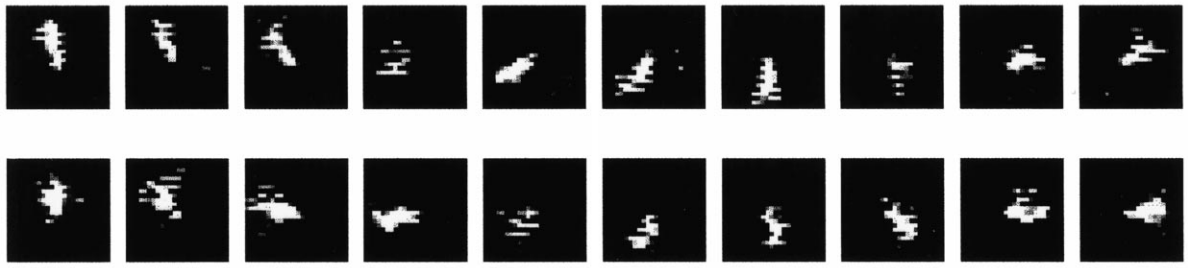

Fig. 2. Sequential images of a rotating actin filament attached to the γ subunit on CF_1 at 20 mM ATP, 5 mM $MgCl_2$. The filament length was 1.26 μm and the averaged rotational speed was 3.2 rounds/s; time interval between images was 33 ms. This figure can be viewed as a time-lapse movie sequence at our web site (http://www.res.titech.ac.jp/seibutu/hisabori/index-e.htm).

which showed unidirectional, counter-clockwise rotation like those on $\alpha_3\beta_3\gamma$ from TF_1 and EF_1 [7–10], when MKR buffer with 8 mM ATP and 2 mM $MgCl_2$, or MKR buffer with 20 mM ATP and 5 mM $MgCl_2$ was infused. At the same time vigorous movements of actin filaments which did not show the continuous rotation were observed. In the midst of the observation, we found that an actin filament suddenly divided into three sections and the middle fragment started wobbling.

The frequency to find the filament which showed the continuous rotation was lower than that for $\alpha_3\beta_3\gamma$ complex of TF_1 [7]. One of the possible reasons might be that the number of the CF_1 derivatives with the top fixed on the glass surface was lower than was the case for TF_1. In the case of TF_1 the orientation of the molecule was controlled by the combination of histidine-tag connected to the β subunit and Ni–nitrilotriacetic acid coated beads on the glass surface [7]. In the case of the CF_1 derivative the orientation was not regular because of the spontaneous adhesion of the molecules on the glass surface.

3.4. The characteristics of rotation of CF_1-γ

As the ATPase activity of $CF_1(-\delta)$ was influenced by the pH of the reaction medium (see above), we tried to observe differences in the rotational speed under various pH conditions. However, we could not find significant differences under our experimental conditions (Fig. 3). One obvious difference to the previous findings on TF_1-$\alpha_3\beta_3\gamma$ [7–10] was a frequently

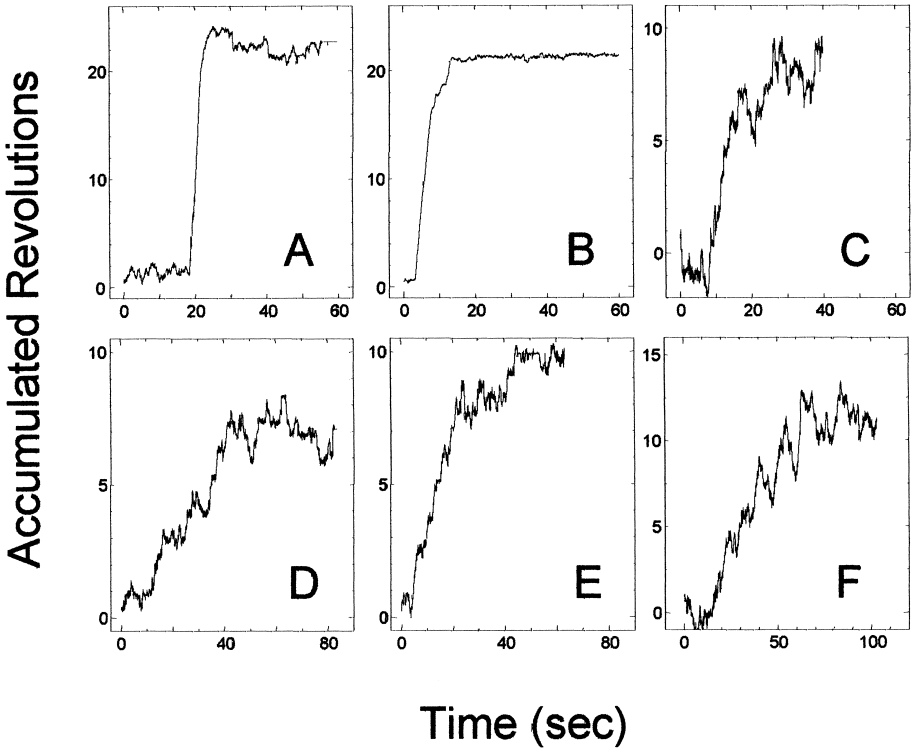

Fig. 3. Time course of the rotation of the γ subunit. Images of the rotating actin filaments were recorded with the intensified CCD camera, and the motion of the centroids was analyzed. The increase of the accumulated revolutions represents the counter-clockwise rotation of the filament. A–C: The rotation of the γ subunit was recorded in the presence of 20 mM ATP, 5 mM $MgCl_2$ at pH 7.0. D and E: The rotation was recorded in the presence of 8 mM ATP, 2 mM $MgCl_2$ at pH 7.0. F: The rotation was recorded in the presence of 8 mM ATP, 2 mM $MgCl_2$ at pH 8.0. The lengths of the observed filaments were 1.26 μm (A), 1.55 μm (B), 1.55 μm (C), 1.58 μm (D), 1.92 μm (E) and 1.46 μm (G), respectively. The filament shown in (E) was propeller type, of which centroid is at the middle of the filament. The filament motion of (E) can be viewed as a time-lapse movie sequence at our web site (http://www.res.titech.ac.jp/seibutu/hisabori/index-e.htm).

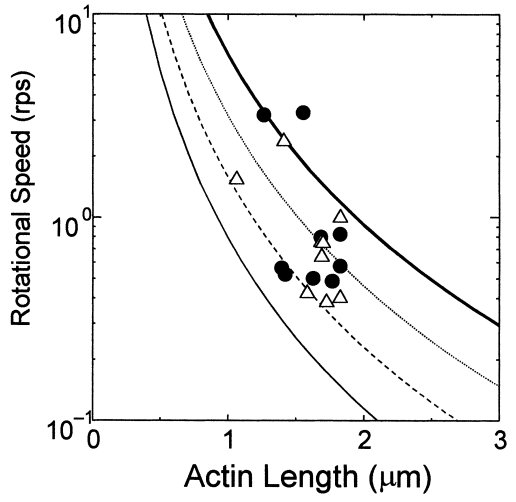

Fig. 4. Rotational speed in revolutions per second versus the length of the actin filament. Filaments that rotated around one end for >5 revolutions without an unusual intermission in the presence of (●) 20 mM ATP or (△) 8 mM ATP were used for the calculation of the rotational speed. Lines show the rotational speed under a constant torque of 40 pNnm (thick line), 20 pNnm (dotted line), 10 pNnm (dashed line) and 5 pNnm (thin line), respectively.

observed vigorous wobble movement of the filaments, which rotated one to two rounds in one and then in opposite direction. Even filament moving in a continuous counter-clockwise rotation (Fig. 3A) suddenly started clockwise movement for a short period. It is not clear yet whether this 'unusual' movement reflects a specific attribute of $CF_1(-\delta)$ compared with the subcomplex of TF_1, or a kind of artifact derived from the different procedure to bind the actin filament on the γ subunit. The different procedure to fix the enzyme molecule on the glass surface may also be the reason.

The highest rotational speed of filaments observed in our experiments was 3.3 rounds/s. As the three catalytic sites on the enzyme are involved in the ATP hydrolysis reaction, this rotational speed implies an ATP hydrolysis reaction with a turnover rate of 10 s^{-1}. This value is 5 to 50-fold higher than the rate obtained by the measurement of ATP hydrolysis activity. The discontinuous motion of the filaments shown in Fig. 3 may explain this apparent discrepancy. Furthermore inhomogeneity of the enzyme preparation, including active and inactive molecules, may also be a possible reason.

By using the highest rotational speed of each of the filaments, we plotted the speed of rotation as a function of the lengths of the actin filaments to roughly estimate the torque (Fig. 4). As in the case of TF_1 [7,8] and EF_1 [9,10], longer actin filaments rotated more slowly than shorter ones. The torque values obtained from the experimental data were within the range of 20–40 pNnm and thus are consistent with the results of previous reports [7–10].

Hence we could observe that the γ subunit of CF_1 can rotate in a similar manner as γ in TF_1 and EF_1 when hydrolyzing ATP. The rotation of the CF_1-γ was already suggested on the basis of fluorescence anisotropy measurements by Sabbert et al. [6]. Our report is, to our knowledge, the first direct observation of γ rotation in an F_1 from a eukaryotic cell although plastids are supposed to be of bacterial origin.

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