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Elastic rotation of *Escherichia coli* F_OF_1 having ε subunit fused with cytochrome b_{562} or flavodoxin reductase

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ABSTRACT

Intra-molecular rotation of F_OF_1 ATP synthase enables cooperative synthesis and hydrolysis of ATP. In this study, using a small gold bead probe, we observed fast rotation close to the real rate that would be exhibited without probes. Using this experimental system, we tested the rotation of F_OF_1 with the ε subunit connected to a globular protein [cytochrome b_{562} (ε-Cyt) or flavodoxin reductase (ε-FlavR)], which is apparently larger than the space between the central and the peripheral stalks. The enzymes containing ε-Cyt and ε-FlavR showed continual rotations with average rates of 185 and 148 rps, respectively, similar to the wild type (172 rps). However, the enzymes with ε-Cyt or ε-FlavR showed a reduced proton transport. These results indicate that the intra-molecular rotation is elastic but proton transport requires more strict subunit/subunit interaction.

1. Introduction

ATP synthase (F_OF_1), ubiquitously found in membranes of bacteria, mitochondria, and chloroplast thylakoids, synthesizes ATP coupled with an electrochemical proton gradient generated by the electron transport chain [1–3]. Bacteria have the simplest version consisting of a peripheral sector F_1 ($\alpha_3\beta_3\gamma\delta\varepsilon$) with three catalytic β subunits, and a membrane integral F_O (ab_2c_{10-15}) with the proton pathway formed from the *a* subunit and the multiple *c* subunits (*c*-ring).

Catalysis and transport are coupled through the intra-molecular rotations, consistent with the "binding change mechanism" [1]. The *c*-ring rotation powered by H^+ transport through two aqueous half-channels and multiple Asp/Glu residues of the *c*-ring supports the sequential conformational changes of catalytic site in each of the three β to synthesize ATP [1–3].

Single molecule observations of *Escherichia coli* F_OF_1 indicated clearly that the $\gamma\varepsilon c_{10}$ complex rotates against the $\alpha_3\beta_3\delta ab2$ subunits during ATP hydrolysis [4–7]. Experimentally, the F_OF_1 was immobilized on the glass surface through the α or β subunit and an actin filament probe attached to the *c* subunit showed counterclockwise rotation [4,6] (Fig. 1A). The probe attached to the β, α, or *a* subunit also indicated the rotation of $\alpha_3\beta_3\delta ab2$ complex against $\gamma\varepsilon c_{10}$, when the purified or membrane bound enzyme was immobilized through the *c*-ring [6,7] (Fig. 1B). However, the rotation rates observed were slow due to the large viscous drag on the actin filament. Experiments using single molecule FRET analysis also showed the rotation of membrane-bound F_OF_1 during ATP synthesis/hydrolysis [8].

Although the detailed tertiary structure of F_OF_1 is still unknown, the structure of mammalian F_1 with its *c*-ring has been reported [9]. The higher-ordered structure obtained by electron microscopy [10] clearly showed the central and peripheral stalks connecting F_1 and F_O, corresponding to the rotor and the stator assembly, respectively. The central stalk was a part of the rotor $\gamma\varepsilon c_{10}$, formed from the γ and the ε subunits and loop regions of the *c* subunits, whereas peripheral stalk was formed from the subunits *b* and δ. The microscopic structure also indicated the presence of an open space between the two stalks [10].

Detailed structures of the ε subunit forming the central stalk were extensively studied. The isolated ε subunit showed two

Abbreviations: $C_{12}E_8$, octaethylene glycol monododecyl ether; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Tricine, *N*-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; Tris, tris(hydroxymethyl)aminomethane; ε-Cyt, ε subunit connected with cytochrome b_{562}; ε-FlavR, ε subunit connected with flavodoxin reductase.

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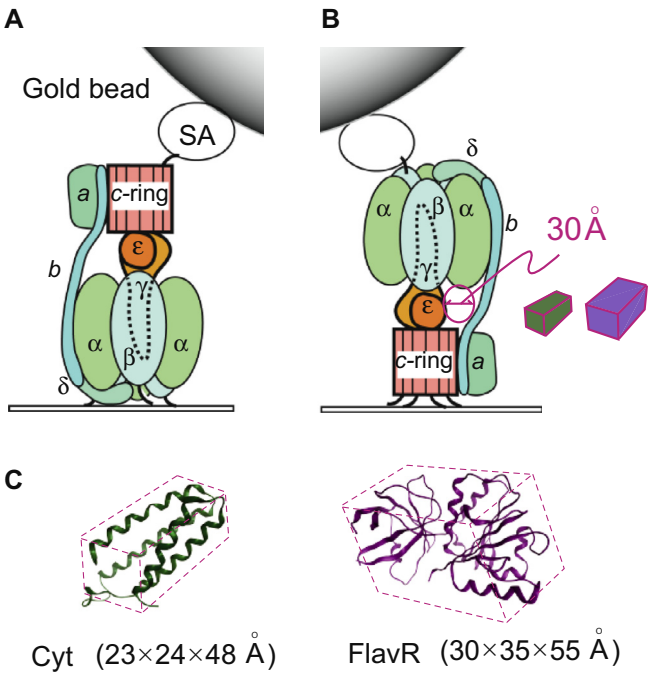

Fig. 1. Rotation observations of the F_OF_1 using 60 nm bead probes. (A) Rotation observation using the gold bead attached to the *c*-ring of F_OF_1. The F_OF_1 was immobilized on the Ni-coated glass via the histidine-tag at the amino termini of the β subunits. The biotinylated gold bead was linked to the *c* subunit through the streptavidin (SA). Upon ATP hydrolysis, the bead revolution was observed. (B) Rotation observation using the gold bead attached to the β subunit of F_OF_1. The F_OF_1 was immobilized through the *c*-ring, and the gold bead attached to the β subunit. The space between the central and the peripheral stalks was estimated [10], and indicated as a sphere space (diameter, ~30 Å). The green and purple cuboids were shown as the fused proteins cytochrome b_{562} and flavodoxin reductase, respectively. (C) Ribbon models of proteins that fused to the carboxyl termini of the ε subunits. Cytochrome b_{562} (23 × 24 × 48 Å) (Cyt, PDB ID: 256B) [27] and Flavodoxin reductase (30 × 35 × 55 Å) (FlavR, PDB ID: 1FDR) [28] are shown.

carboxyl-terminal helices folded near the amino terminal β-sandwich domain [11,12]. However, according to the recent crystal structure of F_1 [13], two ε subunit helices were extended along the coiled-coil of the γ subunit, and the second helix was penetrated between the rotor and the stator to prevent the γ subunit rotation. This structure seemed consistent with the inhibition of F_1 ATPase with the ε subunit [14]. Crosslink studies suggested that the ε subunit adopted both folded and extended conformations in the F_OF_1 [15,16].

Considering the function and structure of the central stalk, it was of interest to study the F_OF_1 carrying the ε subunits fused to globular proteins at the carboxyl terminus [17]. The fused proteins such as cytochrome b_{562}, flavodoxin, and flavodoxin reductase were large enough to affect the enzyme catalysis (Fig. 1C). As expected, the extra moieties caused significantly reduced ATP-driven proton transport while ATPase activities were retained [17]. The wild-type ε subunit inhibited rotation and ATPase activity of F_1 sector, whereas the ε-Cyt (ε subunit connected with cytochrome b_{562}) showed no effect [18], indicating that ε-Cyt lost normal interaction of its carboxyl-terminal region with other subunits. Thus, we concluded that the globular proteins fused to the ε subunits affected rotation, leading to lower proton transport. However, no studies were carried out to address the effect of ε-Cyt on rotational catalysis of F_OF_1.

In this study, we observed faster rotation of the F_OF_1 with a gold bead probe (60 nm) attached to the β subunit or the *c*-ring (Fig. 1A, B). Since viscous drag on the revolving small bead was substantially low, rotation rates observed were 30–50-fold higher than that with actin probe, and close to the rate which would be exhibited without a probe. This experimental system prompted us to test the rotation of F_OF_1 with the ε subunit connected to the extra globular proteins because they reduced energy coupling to H^+-pumping. Surprisingly, the beads attached to the β subunit of the enzymes containing the ε-Cyt and the ε-FlavR showed rates similar to that of the wild-type enzyme. Considering the dimensions of the extra domains included into the $\gamma\varepsilon c_{10}$ complex, these studies suggested that the F_OF_1 has elasticity which permits rotation of the large central stalk.

2. Materials and methods

2.1. Recombinant plasmids

F_OF_1 operon containing the genes for histidine-tagged β subunit and the *c* subunit with *c*Glu2Cys substitution was previously described [6]. Genes for the ε-fusions were introduced into pBUR13DX, a derivative of pBWU13 [4], carrying all F_OF_1 subunit genes with the sequences for the biotin- and the histidine-tags at the amino termini of the β and the *c* subunit genes, respectively [19]. Using *Sac*I site in the β subunit gene and newly introduced *Xba*I site 323 bp downstream of the termination codon of the ε subunit gene, the sequences for the ε-Cyt and the ε-FlavR (formerly named as ε-Red and ε-Yellow, respectively) [17] were introduced. Genes for the ε-fusions with a linker sequence between the ε and the fusion proteins (ε-L-Cyt and ε-L-FlavR) [17] were also introduced.

2.2. Preparation of F_OF_1

Recombinant plasmids were introduced into the *E. coli* strain DK8 (Δ*atp*B-C) [20]. Membrane vesicles (containing about 40 mg of proteins) prepared after disruption of the cells grown on glycerol were suspended in 3.2 ml of Buffer A [40 mM MES-Tricine (pH7.0 at 25°C), 10 mM $MgCl_2$ and 20%(w/v) glycerol] then solubilized by addition of 0.8 ml of 10%(w/v) $C_{12}E_8$ (final concentration of 2%). The suspension was centrifuged at 125,000×*g* for 60 min, and the supernatant was slowly applied to the Ni-nitrilotriacetic acid agarose column (0.6 × 1.5 cm, Qiagen) equilibrated with Buffer B [20 mM MES-Tricine (pH7.0 at 25 °C), 5 mM $MgCl_2$, 10% glycerol and 2% $C_{12}E_8$]. The column was washed with 6 ml of Buffer C [20 mM MES-Tricine (pH7.0 at 25 °C), 5 mM $MgCl_2$, 10% glycerol, 0.1% $C_{12}E_8$, 0.03% (w/v) L-α-phosphatidylcholine and 20 mM imidazole]. F_OF_1 was eluted with the same buffer by increasing the imidazole concentration up to 200 mM dialyzed against Buffer C containing 25% glycerol. All procedures described above were carried out at 4 °C. Purified enzyme was quickly frozen in liquid nitrogen and stored at −80 °C until use.

2.3. DCCD sensitivities of membrane and F_OF_1 ATPase activities

Membranes (20 μg protein) or purified F_OF_1 (4 μg of protein) were treated in 100 μl of 50 mM Tris–HCl (pH8.0) buffer containing 40 μM DCCD (*N*,*N*′-dicyclohexylcarbodiimide), 2 mM $MgCl_2$, 300 mM KCl for 15 min at 22 °C. ATPase activity of 50 μl aliquot was assayed at 22 °C with coupled NADH oxidation in the presence of ATP regeneration system [21].

2.4. Rotation observation of immobilized enzymes

Rotations of both F_OF_1 molecules immobilized through the β subunits and the *c*-ring were observed using essentially the same procedures for F_1 rotations as described previously [18]. Briefly, a flow cell was filled with Buffer D [10 mM MOPS-KOH, 50 mM