Stochastic rotational catalysis of proton pumping F-ATPase

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F-ATPases synthesize ATP from ADP and phosphate coupled with an electrochemical proton gradient in bacterial or mitochondrial membranes and can hydrolyse ATP to form the gradient. F-ATPases consist of a catalytic F₁ and proton channel F₀ sectors, respectively. The rotation of the catalytic hexamer, 120° stepped revolution has been observed, each step being divided into two substeps. The ATP-dependent revolution exhibited stochastic fluctuation and was driven by conformation transmission of the β subunit (phosphate-binding P-loop/α-helix B/loop/β-sheet). Recent results regarding mechanically driven ATP synthesis finally proved the role of rotation in energy coupling.

Keywords: F-ATPase; ATP synthase; rotational catalysis; F₁; F₀

1. INTRODUCTION

Cellular ATP is synthesized by a proton pumping F-ATPase (ATP synthase) coupled with an electrochemical proton gradient generated by the respiratory chain. The entire mechanism (oxidative- or photophosphorylation) is carried out in the membranes of mitochondria, bacteria or chloroplasts. F-ATPase, named after coupling factors of phosphorylation, is carried out in the membranes of chloroplasts, bacteria or chloroplasts. F-ATPase (ATP synthase) coupled with an electrochemical proton gradient in bacterial or mitochondrial membranes and can hydrolyse ATP to form the gradient. F-ATPases follow the binding change mechanism (Boyer 1997): briefly, ADP and Pi (phosphate) bind to a loose site that changes the conformation mechanism (Boyer 1997): briefly, ADP and Pi (phosphate) bind to a loose site that changes the conformation mechanism (Boyer 1997): briefly, ADP and Pi (phosphate) bind to a loose site that changes the conformation of the site to a tight one for ATP synthesis, while ATP is released from another site. The mechanism proposes that the reaction ADP + Pi → ATP + H₂O at the tight site is reversible with no energy change, and that three sites are involved in the ATP synthesis sequentially through rotation of the catalytic β hexamer as to the γ subunit.

2. CATALYSIS, TRANSPORT AND ENERGY COUPLING BY F-ATPASES

(a) Catalysis by F-ATPases

F-ATPases couple proton transport in F₀ and chemistry in F₁ through mechanical rotation. As expected from the presence of the three catalytic β subunits in an αβ₁ hexamer, F-ATPases follow the binding change mechanism (Boyer 1997): briefly, ADP and Pi (phosphate) bind to a loose site that changes the conformation of the site to a tight one for ATP synthesis, while ATP is released from another site. The mechanism proposes that the reaction ADP + Pi → ATP + H₂O at the tight site is reversible with no energy change, and that three sites are involved in the ATP synthesis sequentially through rotation of the catalytic β hexamer as to the γ subunit.

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Figure 1. Schematic illustration of F-ATPases (F₁ and F₀ sectors). ATP synthesis/hydrolysis is coupled with proton transport through mechanical rotation. The number of c subunit monomers varies between 10 and 14 depending on the source. The model is for E. coli or yeast F-ATPase with a ring formed from 10 monomers.

$10^{5}$–$10^{6}$ fold faster than that of the ATP hydrolysis at a single site assayed with an ATP : F₁ ratio of less than 1 : 3 (Cross et al. 1982). Further kinetic studies indicated that the equilibrium constant at the catalytic site is near unity, supporting that ATP is synthesized with no energy change (Grubmeyer et al. 1992). Three asymmetric catalytic sites were suggested by kinetic studies on wild-type and mutant enzymes, nucleotide binding binding being detected with an intrinsic tryptophan probe, affinity labelling with ATP analogues or chemical modification with inhibitors (Boyer 1997; Senior et al. 2002; Futai et al. 2003). Consistent with the asymmetric mechanism, the X-ray structure showed three different catalytic sites in $\beta_{b}$, $\beta_{DP}$ and $\beta_{TP}$ corresponding to empty, ADP- and ATP-bound $\beta$ subunits, respectively (Abrahams et al. 1994).

Catalytic residues were identified in the E. coli F₁ (Ida et al. 1991; Omote et al. 1992; Senior & Al-Shawi 1992; Senior et al. 1993; Park et al. 1994; Łóbau et al. 1997; figure 2). $\beta$Lys155 of the $\beta$ subunit is required for the binding of the $\gamma$ phosphate moiety of ATP, as shown by enzymes such as the ones with $\beta$Lys155 changed to Ala, Ser, Thr, Gln or Glu, and the affinity labelling of ATP analogues. The hydroxyl moiety of $\beta$Thr156 is possibly essential for Mg²⁺ binding. $\beta$Glu181 is a critical catalytic residue forming a hydrogen bond with a water molecule near the ATP $\gamma$ phosphate.

Analysis of intrinsic tryptophan probe ($\beta$Tyr331Trp) indicated that the $\beta$Lys155, $\beta$Glu181 and $\beta$Asp242 are catalytic residues: $\beta$Lys155 interacts with MgATP, $\beta$Glu181 is a major catalytic residue and $\beta$Asp242 interacts with magnesium (Łóbau et al. 1997). $\beta$Glu185 (Omote et al. 1995) and $\alpha$Arg376 (Le et al. 2000) of the $\beta$ and $\alpha$ subunits, respectively, are required for catalytic cooperativity. These residues could be located near ATP in the crystal structure of F₁ (figure 2; Abrahams et al. 1994) and the transition state of the catalytic site (Braig et al. 2000).

(b) F₀ sector and proton pathway

Subunit $\alpha$ has five transmembrane helices and the conserved essential $\alpha$Arg210 is in the fourth helix (Wada et al. 1999), close to the amino-terminal helix of the $c$ subunit: a model of the interaction of the two helices was discussed (Fillingame et al. 2000). The $b$ subunit is a long helical protein whose amino-terminal is embedded in the membrane, and its carboxyl terminus interacts with $\alpha$ and $\delta$ at the top of F₁ (Fillingame et al. 2000). The structure of the $c$ subunit, two transmembrane $\alpha$-helices connected by a polar loop, has been solved by NMR (Girvin et al. 1998). Low-resolution electron (Birkenhäuser et al. 1990) and atomic force microscopy (AFM; Singh et al. 1996; Takeyasu et al. 1996) suggested a ring structure formed from multiple $c$ subunits. Refined AFM studies indicated that chloroplast and bacterial (Rhyobacter tartaricus) rings comprise 14 (Seelert et al. 2000) and 11 (Stahlberg et al. 2001) monomers, respectively. The X-ray structure of yeast F₁ with a 10 monomer ring was solved (Stock et al. 1999). The X-ray diffraction has recently revealed an 11 monomer ring of I. tartaricus F-ATPase (Meier et al. 2005) and a 10 monomer ring of Enterococcus hirae V-ATPase (Murata et al. 2005). Cross-linking studies on the E. coli F-ATPase are consistent with 10 monomers, with amino and carboxyl helical domains located inside and outside, respectively (Fillingame et al. 2000).

Functional $c$ rings were suggested by expressing covalently fused E. coli or Bacillus genes (Jiang et al. 2001; Mitome et al. 2004). The 10 oligomer formed from the fused genes was active. A similar experiment was carried out by nature: a gene encoding 13 homologous domains of the $c$ subunits covalently connected was found in archae Methanopyrus kandleri (Lolkema & Boekema 2003). These results established a $c$ subunit ring structure, the number of monomers differing with the species.

cAsp61, at the middle of the second transmembrane helix of the E. coli c subunit, is responsible for proton transport, and the results of mutational studies are consistent with rotary movement of the $c_{10}$ ring (Fillingame & Dmitriev 2002). The stoichiometry (1 : 10–14) of the $a$ and $c$ subunits indicates that one $a$Arg210 sequentially interacts with multiple carboxyl moieties for continuous proton translocation.
(c) Role of the \( \gamma \) subunit in energy coupling

An essential role of the \( \gamma \) subunit in catalysis and assembly was suggested by early experiments including reconstitution of the \( \alpha_3\beta_3\gamma \) complex with ATPase activity (Dunn \& Futai 1980) and analysis of defective termination mutants (Iwamoto et al. 1990). A role of chloroplast \( \gamma \) in regulation was indicated by the presence of a unique domain for reversible formation of a disulfide bond (Miki et al. 1988). Although the sequences of \( \gamma \) are weakly homologous among species, the amino- and carboxyl-terminal regions are significantly conserved (Nakamoto et al. 1992).

Of the mutants suggesting an active role of the \( \gamma \) subunit, \( \gamma \)Met23→Lys and \( \gamma \)Met23→Arg substitutions (Shin et al. 1992) should be briefly mentioned. They are impaired in ATP synthesis, and can only form a low electrochemical proton gradient dependent on ATP hydrolysis. The \( \gamma \)Met23 residue is located close to the DELSEED loop (\( \beta Asp380–\beta Asp386 \)) of \( \beta \). Thermodynamic studies suggested that the mutant Lys23 could form an ionized hydrogen bond with \( \beta \)Glu381 of one of the three \( \beta \) subunits (Al-Shawi et al. 1997). These results suggest that the interaction between the region including \( \gamma \)Met23 and DELSEED is involved in energy coupling.

The defect of the Lys23 mutant was suppressed by a second mutation in the carboxyl-terminal region of the \( \gamma \) subunit (Nakamoto et al. 1993, 1995). Most of the second mutations were mapped at positions far from position 23, but near the region where \( \gamma \) could interact with \( \beta \). These results suggested that the \( \alpha \)-helices of \( \gamma \) located at the centre of the \( \alpha_3\beta_3 \) hexamer undergo large conformational changes during catalysis, as expected from the different orientation of \( \gamma \) as to the three \( \beta \) (\( \beta b \), \( \beta_{DP} \) and \( \beta_{TP} \); Abrahams et al. 1994) subunits.

3. ROTATIONAL CATALYSIS OF F-ATPASE

(a) \( \gamma \) subunit rotation in the \( F_i \) sector

The binding change mechanism predicted that the catalytic sites in the three \( \beta \) subunits sequentially participate in ATP synthesis or hydrolysis through conformational transition via rotation of the \( \gamma \) located at the central space of the \( \alpha_3\beta_3 \). Observing rotation became possible using available X-ray structures (Abrahams 1994), since an appropriate probe could be introduced at a defined position of immobilized \( F_i \) or \( F_0F_1 \). Rotation of \( \gamma \) was suggested by cross-linking between \( \gamma \)Cys87 and \( \beta \)Cys380 in the DELSEED loop (\( Asp380–Asp386 \)) of \( E. \) coli \( F_i \) (Duncan et al. 1995), and analysis of polarized absorption recovery after photobleaching of a probe linked to the carboxyl terminus of chloroplast \( F_i \) (Sabbert et al. 1996).

The rotation of \( \gamma \) was unambiguously video recorded by Noji et al. (1997). They observed ATP-dependent rotation of an actin filament connected to \( \gamma \) of the immobilized the Bacillus \( \alpha_3\beta_3\gamma \) complex. The anticlockwise revolution speed became slower with an increase in filament length, generating frictional torque of approximately 40 pN·nm. The rotation of the \( \epsilon \) subunit was shown subsequently (Kato-Yamada et al. 1998).

(b) Subunit rotation in the F-ATPase holoenzyme

Revolution of \( \gamma \) should be transmitted to the \( F_i \) during ATP-dependent proton translocation in F-ATPase. Assuming that \( \gamma \) rotates with the \( c \) ring, purified F-ATPase was immobilized through the \( \alpha \) subunit. An actin filament connected to the \( c \) ring rotated upon ATP hydrolysis and generated similar torque to that observed for the \( \gamma \) rotation (Sambongi et al. 1999). A similar experiment was carried out using a different method to connect the actin probe to the \( c \) ring (Pánke et al. 2000). Experimental systems for observing rotation were critically discussed (Sambongi et al. 2000; Wada et al. 2000). These results suggest that \( \gamma \), \( \epsilon \) and the \( c \) ring form a rotor, consistent with the finding that cross-linking between \( \gamma \) and \( \epsilon \) or \( \epsilon \) and \( c \) did not affect ATPase activity (Schulenberg et al. 1999). Obviously, the cross-linking between rotor and stator such as \( \beta \) and \( \gamma \) or \( \beta \) and \( \epsilon \), respectively, resulted in the loss of the activity (Aggerel et al. 1999).

Furthermore, an actin filament connected to the \( \alpha \) or \( \alpha \) subunit rotated upon ATP hydrolysis in F-ATPase immobilized through the \( c \) ring (Tanabe et al. 2001). Similar results were obtained with membrane-bound F-ATPase, which was not subjected to solubilization with a detergent (Nishio et al. 2002). The rotation of gold beads attached to the Bacillus \( F_0F_1 \) \( c \) ring was shown recently, the rates being approximately 300 revolutions per second (rps) at 37°C (Ueno et al. 2005). These results established that the \( \gamma \)FG10 and \( \alpha_3\beta_3\delta_2 \) complexes are an interchangeable rotor and a stator, respectively.

(c) Rotational synthesis of ATP

Chemiosmotic theory had been supported by ATP synthesis driven by an electrochemical proton gradient applied artificially (Mitchell 1979). Subunit rotation during ATP synthesis was shown recently with an F-ATPase in liposomes by fluorescence resonance energy transfer analysis (Diez et al. 2004). Thus, the electrochemical gradient should rotate the \( \gamma \)FG10 in F-ATPase, followed by ATP synthesis at the \( \beta \) subunit catalytic site.

ATP synthesis driven by the artificial \( \gamma \) revolution was shown (Itoh et al. 2004). Rotation of a bead in the magnetic field to the clockwise direction (viewed from the membrane) resulted in ATP synthesis. Rondelez et al. (2005) also observed ATP synthesis when \( \gamma \) was rotated artificially. Further studies will address the mechanistic questions including whether or not the rotation speed is proportional to the torque applied, or revolution is initiated upon applying torque higher than a certain value.

4. STEPPING STOCHASTIC ROTATION

(a) Stepping rotation

As predicted by the sequential catalysis at the three sites, three 120° steps of Bacillus \( F_1 \) were observed using an actin filament, when the ATP concentration was lowered (Yasuda et al. 1998). The stepping rotation was confirmed using beads with low viscous drag
Furthermore, the stepping was observed with ATP concentrations of approximately 30-fold higher than the Km values (Yasuda et al. 2001; Nakanishi-Matsui et al. 2007). Further analysis using smaller probes has shown that each step is divided into 90 and 30° substeps (Yasuda et al. 2001), later revised to 80 and 40°, respectively (Shimabukuro et al. 2003). They were attributed to ATP binding and phosphate release, respectively (Adachi et al. 2007). Thus, the rotation pauses before the 80° step, when the ATP concentration was lowered. The 120° steps observed with a higher ATP concentration most probably reflected ATP hydrolysis/product release (Nakanishi-Matsui et al. 2007).

**Stochastic rotation**

The maximal speed of an actin filament connected to the γ subunit was approximately 10 rps (Sabbert et al. 1996; Noji et al. 1997; Omote et al. 1999), i.e. slower than the rate expected from the turnover number of the steady-state ATPase. The ATPase rate calculated from the filament rotation was approximately 30 s⁻¹, assuming that three ATP molecules were hydrolysed for one revolution (Nakanishi-Matsui et al. 2006). A gold bead attached to γ rotated faster (figure 3), possibly at a rate close to that of γ without a probe, since ones of 40 and 60 nm diameter rotated essentially at the same speed. The average rate during 250 ms observation was approximately 380 rps, i.e. approximately 10 times faster than the value expected from the ATPase. These results suggested that approximately 10% of the F₁ molecules were rotating at the milliseconds time resolution. This interpretation became more convincing on observing longer time courses of randomly selected beads (Nakanishi-Matsui et al. 2007): individual beads paused randomly and rotated again (figure 4a). Histograms of the rotation speed indicated that the rotation exhibited stochastic fluctuation (figure 4b) essentially independent of the probe sizes (40–200 nm diameter; Nakanishi-Matsui & Futai 2006).
When the beads were followed for a longer time, we observed that they unexpectedly paused sometimes for longer than 0.1 s, possibly due to MgADP inhibition (Nakanishi-Matsui et al. 2007). Longer pause of single beads should certainly lower the steady-state ATPase, which corresponds to the average rate of randomly selected beads.

The γ rotated in a stepwise manner, indicating that overall speed depends on the pausing dwells (approx. ms) and the time required for revolution between the steps (0 → 120°, 120 → 240°, 240 → 360° per 0°). The time required for the 120° revolution was mostly ≤ 0.25 ms, whereas pausing dwell was longer and variable. Thus, we used a single revolution time, i.e. the time for the 360° revolution, to analyse bead rotation with variable rates (figure 4c). This parameter could express all revolutions of a bead, even those including long pauses in a single figure. The histograms of the single revolution time for each bead and those of multiple beads combined were similar.

The fluctuation of the rotation rate was independent of the probe size (40–200 nm; figure 3b) when normalized (Nakanishi-Matsui & Futai 2006). Rotation of a single fluorophore attached to the γ subunit also exhibited variable dwelling times (Adachi et al. 2000). We have observed similar fluctuation of gold beads rotation in F₀F₁ (in preparation). These results indicate that the fluctuation is an intrinsic property of F-ATPase.

The effect of the ε subunit, an inhibitor of F₁ ATPase, on the rotation was clearly observed when a gold bead was used as the probe (Nakanishi-Matsui et al. 2006). Histograms of the rates in the presence of excess ATP showed two populations, one peak at less than 40 rps and another at 200–240 rps. Most of the former peak was due to increased pausings, and the second peak was slower than that without ε. These results indicated that ε is inhibitory by increasing the pausing duration.

5. TOWARDS UNDERSTANDING THE ROTATION MECHANISM

(a) Mutation studies of rotation

The mechanisms of catalysis and energy coupling of F-ATPase have been studied by analysing the mutants (Futai et al. 1989, 2003). Substitution of βSer174 had interesting effects on ATPase activity: the larger the side-chain volume of the residue introduced, the lower the ATPase activity became (Omote et al. 1994), and F₁ with Phe or Leu exhibited approximately 10% wild-type activity. The introduced residues may affect the conformation of the β-sheet4 and the α-helix B domain (figure 5a), because introduced Phe may interact with the βIle166 or βIle163 residue in α-helix B (Iko et al. 2001).

The defect caused by βPhe174 was suppressed by a second mutation replacing βGly149 in the phosphate-binding P-loop (Iwamoto et al. 1993), indicating that the interaction between β-sheet4 and P-loop is important for ATPase activity. However, torque generated by an actin filament connected to βPhe174 or βLeu174 did not correspond to the ATPase activity, suggesting that actin is not suitable for studying mutant F₁ (Iko et al. 2001).

Gold beads were attached to the mutant γ subunit (Nakanishi-Matsui et al. 2007), expecting that they reflect rotation as the speed of the γ subunit. Similarly, in the case of the wild-type, the single revolution time of a 60 nm gold bead showed stochastic fluctuation. Both βPhe174 and βLeu174 exhibited longer single revolution time than the wild-type (figure 5b), whereas those with a second mutation (βGly149Ala) were essentially similar to the wild-type. The βGly149 residue is in the P-loop where catalytic residues such as βLys155 and βThr156 are present (figure 2). These results suggest that the conformational transmission between the P-loop and β-sheet4 should be an initial change for driving rotation.

We were interested in the γLys23 mutation, which causes a defect in energy coupling between ATPase and proton translocation (Shin et al. 1992). Using an actin filament, we observed that the F₁ generated essentially the same torque regardless of the mutation (Omote et al. 1999), suggesting that the mutant γMer23Lys is defective in transforming mechanical work into proton transport. However, the mutant's rotation should be studied using probe with lower viscous drag. Furthermore, it is of interest to examine the effect of replacing cAsp61 or aArg210 on rotation, which impairs proton transport (Hosokawa et al. 2005).

(b) For understanding the rotation mechanism

It has been accepted that F-ATPase couples proton transport and ATP synthesis/hydrolysis through subunit rotation. An important concept is stochastic fluctuation of F-ATPase rotation. The H⁺/ATP ratio

Figure 5. β subunit domain essential for F₁ rotation. (a) β Subunit domain between β-sheet4 and P-loop. A β subunit domain is shown for an (i) empty (βP) β subunit or (ii) ATP bound (βTP). The positions of Ser174, Gly149 and ATP are shown according to the bovine F₁ (Abrahams et al. 1994). (b) Effects of the βPhe174 mutation and a second mutation (βGly149Ala) on rotation. Fitted curves were obtained from the histogram of the single revolution time of the wild-type and mutant F₁ (wild-type, black solid curve; Phe174, grey solid curve; Phe174/Ala149, grey dashed curve; and Ala149, black dashed curve).
for each step may also be stochastic. As discussed above, F1 has three catalytic sites, whereas proton transporting aspartate or glutamate in F0 has number 10, 11, 13 or 14 depending on the source. Assuming that all carboxyl moieties are used and three ATP molecules are synthesized or hydrolysed in one 360° revolution, the H+/ATP ratio should be 3.3, 3.7, 4.3 and 4.6 for the different species. To accommodate the non-integer H+/ATP ratio, the number of protons transported in each 120° step may be variable: for example, three protons in two 120° steps and four protons in one 120° step may be transported in E. coli F-ATPase having a c ring of 10 monomers (figure 1).

An enzyme is generally defined with kinetic parameters such as Km and Vmax obtained from steady-state kinetics. However, each enzyme molecule should exhibit variable rates, as shown for the rotation of a bead connected to F1. It should be noted that kinetic parameters determined on bulk phase measurement for ATPase are the averages for individual molecules.

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