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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 F_1 and proton channel F_0 formed from the $\alpha_3\beta_3\gamma\delta\varepsilon$ and ab_2c_{10} subunit complexes, respectively. The rotation of $\gamma\varepsilon c_{10}$ couples catalyses and proton transport. Consistent with the threefold symmetry of the $\alpha_3\beta_3$ 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-sheet4). Recent results regarding mechanically driven ATP synthesis finally proved the role of rotation in energy coupling.

Keywords: F-ATPase; ATP synthase; rotational catalysis; F_1; F_0

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 (Pedersen & Carafoli 1987), is a membrane enzyme formed from a catalytic sector, F_1, and a proton pathway, F_0 (figure 1). The basic structure of F_1 is formed from the α, β, γ, δ and ε subunits with a stoichiometry of 3 : 3 : 1 : 1 : 1, and that of F_0 from the *a*, *b* and *c* subunits (1 : 2 : 10–14).

Taking advantage of its stability, the *Bacillus* F-ATPase formed from the TF_1 and TF_0 sectors (T for thermophilic) was purified and reconstituted (Sone *et al.* 1975; Yoshida *et al.* 1975). The reconstituted F-ATPase could generate an electrochemical proton gradient upon ATP hydrolysis, and, in the reverse direction, could synthesize ATP coupled with the gradient (Sone *et al.* 1977). These results biochemically defined F-ATPase as a chemiosmotic enzyme (Mitchell 1979).

Functional F_1 was obtained from *Escherichia coli* (Futai *et al.* 1974) and reconstituted from the five isolated subunits (Dunn & Futai 1980). The subunits of F_0 and F_1 were determined from the transducing λ phage carrying the entire genes for F-ATPases (Foster *et al.* 1980). F-ATPase purified from an overproducing strain could be functionally reconstituted in liposomes (Moriyama *et al.* 1991). *Escherichia coli* F-ATPase is the first enzyme whose genes and subunit sequences were determined (Kanazawa & Futai 1982; Futai & Kanazawa 1983; Walker *et al.* 1984) and has been studied with genetic manipulation.

X-ray structure of bovine F_1 (Abrahams *et al.* 1994) clearly supported the binding change mechanism for ATP synthesis, including mechanical rotation of subunits (Boyer 1997). It became possible to correlate mutational results to the higher ordered structure.

In this article, discussion is focused on rotational catalysis and energy coupling by F-ATPases. We apologize to those whose works are not cited due to space limitation. For the areas not discussed in detail, readers could refer to the reviews (Futai *et al.* 1989, 2003; Stock *et al.* 2000; Senior *et al.* 2002; Fillingame *et al.* 2003). Other reviews are cited elsewhere where it is appropriate.

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

(a) *Catalysis by F-ATPases*

F-ATPases couple proton transport in F_0 and chemistry in F_1 through mechanical rotation. As expected from the presence of the three catalytic β subunits in an $\alpha_3\beta_3$ 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 + H_2O' 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 $\alpha_3\beta_3$ hexamer as to the γ subunit.

ATP hydrolysis in the F_1 can be measured under single site (unisite catalysis) or steady-state (multisite catalysis) conditions: the steady-state ATPase rate is

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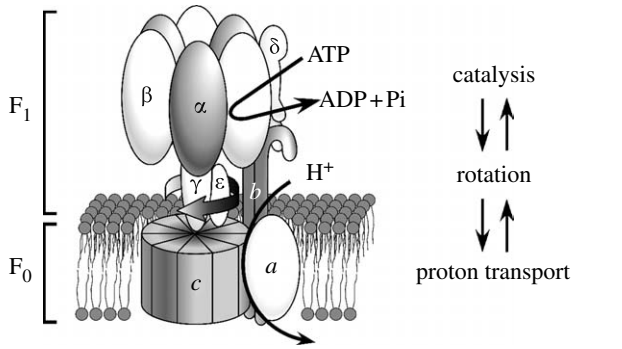

Figure 1. Schematic illustration of F-ATPases (F_1 and F_0 sectors). ATP synthesis/hydrolysis is coupled with proton transport through mechanical rotation. The number of *c* subunit monomers varies between 10 and 14 depending on the source. The model is for *E. coli* or yeast F-ATPase with a ring formed from 10 monomers.

10^5–10^6 fold faster than that of the ATP hydrolysis at a single site assayed with an ATP : F_1 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.* 1982). Three asymmetric catalytic sites were suggested by kinetic studies on wild-type and mutant enzymes, nucleotide 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 β_E, β_{DP} and β_{TP} corresponding to empty, ADP- and ATP-bound β subunits, respectively (Abrahams *et al.* 1994).

Catalytic residues were identified in the *E. coli* F_1 (Ida *et al.* 1991; Omote *et al.* 1992; Senior & Al-Shawi 1992; Senior *et al.* 1993; Park *et al.* 1994; Löbau *et al.* 1997; figure 2). βLys155 of the β subunit is required for the binding of the γ phosphate moiety of ATP, as shown by enzymes such as the ones with βLys155 changed to Ala, Ser, Thr, Gln or Glu, and the affinity labelling of ATP analogues. The hydroxyl moiety of βThr156 is possibly essential for Mg^{2+} binding. βGlu181 is a critical catalytic residue forming a hydrogen bond with a water molecule near the ATP γ phosphate.

Analysis of intrinsic tryptophan probe (βTyr331Trp) indicated that the βLys155, βGlu181 and βAsp242 are catalytic residues: βLys155 interacts with MgATP, βGlu181 is a major catalytic residue and βAsp242 interacts with magnesium (Löbau *et al.* 1997). βGlu185 (Omote *et al.* 1995) and α Arg376 (Le *et al.* 2000) of the β and α subunits, respectively, are required for catalytic cooperativity. These residues could be located near ATP in the crystal structure of F_1 (figure 2; Abrahams *et al.* 1994) and the transition state of the catalytic site (Braig *et al.* 2000).

(b) *F_0 sector and proton pathway*

Subunit *a* has five transmembrane helices and the conserved essential *a*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

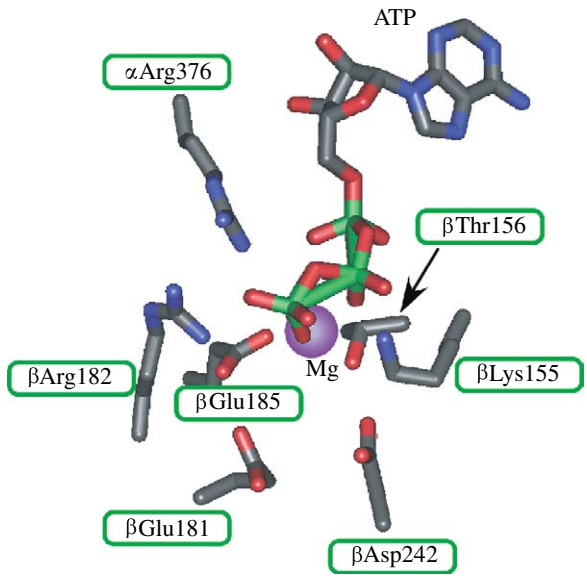

Figure 2. Catalytic site of F-ATPases. The catalytic site of *E. coli* F-ATPase is shown together with bound ATP. The amino acid positions are according to the bovine F_1 (Abrahams *et al.* 1994).

b subunit is a long helical protein whose amino-terminal is embedded in the membrane, and its carboxyl terminus interacts with α and δ at the top of F_1 (Fillingame *et al.* 2000). The structure of the *c* subunit, two transmembrane α-helices connected by a polar loop, has been solved by NMR (Girvin *et al.* 1998). Low-resolution electron (Birkenhäger *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 (*Ilyobacter tartaricus*) rings comprise 14 (Seelert *et al.* 2000) and 11 (Stahlberg *et al.* 2001) monomers, respectively. The X-ray structure of yeast F_1 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 γ subunit in energy coupling*

An essential role of the γ 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 γ 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 γ 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 γ subunit, γMet23→Lys and γMet23→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 γMet23 residue is located close to the DELSEED loop (βAsp380–βAsp386) of β. Thermodynamic studies suggested that the mutant Lys23 could form an ionized hydrogen bond with βGlu381 of one of the three β subunits (Al-Shawi *et al.* 1997). These results suggest that the interaction between the region including γMet23 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 γ subunit (Nakamoto *et al.* 1993, 1995). Most of the second mutations were mapped at positions far from position 23, but near the region where γ could interact with β. These results suggested that the α-helices of γ located at the centre of the $\alpha_3\beta_3$ hexamer undergo large conformational changes during catalysis, as expected from the different orientation of γ as to the three β (β_E, β_{DP} and β_{TP}; Abrahams *et al.* 1994) subunits.

3. ROTATIONAL CATALYSIS OF F-ATPASE

(a) *γ subunit rotation in the F_1 sector*

The binding change mechanism predicted that the catalytic sites in the three β subunits sequentially participate in ATP synthesis or hydrolysis through conformation transmission via rotation of the γ 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_1 or F_0F_1. Rotation of γ was suggested by cross-linking between γCys87 and introduced βCys380 in the DELSEED loop (Asp380–Asp386) of *E. coli* F_1 (Duncan *et al.* 1995), and analysis of polarized absorption recovery after photobleaching of a probe linked to the carboxyl terminus of chloroplast F_1 (Sabbert *et al.* 1996).

The rotation of γ was unambiguously video recorded by Noji *et al.* (1997). They observed ATP-dependent rotation of an actin filament connected to γ 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 ε subunit was shown subsequently (Kato-Yamada *et al.* 1998). Using an actin probe, we confirmed *E. coli* γ subunit rotation sensitive to F_1 ATPase inhibitor azide, and generated torque of approximately 40 pN·nm (Omote *et al.* 1999). This finding prompted us to study the rotation of the F-ATPase holoenzyme and its mechanism by analysing previously isolated mutants.

(b) *Subunit rotation in the F-ATPase holoenzyme*

Revolution of γ should be transmitted to the F_0 during ATP-dependent proton translocation in F-ATPase. Assuming that γ rotates with the *c* ring, purified F-ATPase was immobilized through the α subunit. An actin filament connected to the *c* ring rotated upon ATP hydrolysis and generated similar torque to that observed for the γ rotation (Sambongi *et al.* 1999). A similar experiment was carried out using a different method to connect the actin probe to the *c* ring (Pänke *et al.* 2000). Experimental systems for observing rotation were critically discussed (Sambongi *et al.* 2000; Wada *et al.* 2000). These results suggest that γ, ε and the *c* ring form a rotor, consistent with the finding that cross-linking between γ and *c* or ε and *c* did not affect ATPase activity (Schulenberg *et al.* 1999). Obviously, the cross-linking between rotor and stator such as β and γ or β and ε, respectively, resulted in the loss of the activity (Aggeler *et al.* 1999).

Furthermore, an actin filament connected to the *a* or α 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 $\gamma\varepsilon c_{10}$ and $\alpha_3\beta_3\delta ab_2$ 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 $\gamma\varepsilon c_{10}$ in F-ATPase, followed by ATP synthesis at the β subunit catalytic site.

ATP synthesis driven by the artificial γ 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 γ 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 stepped rotation was confirmed using beads with low viscous drag

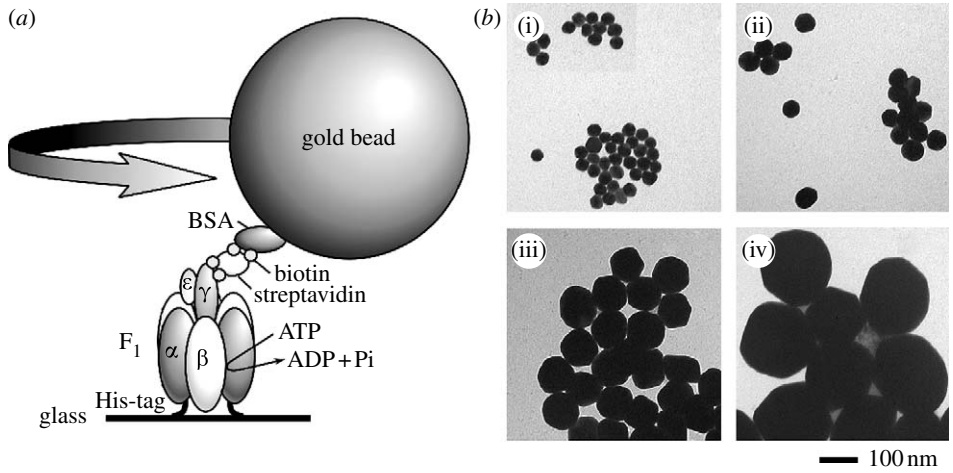

Figure 3. Rotation of gold beads attached to the γ subunit of F_1. (*a*) Experimental set-up for observing gold bead rotation. F_1 was immobilized on a glass surface, a gold bead was attached and ATP-dependent rotation was followed. (*b*) Electron microscopy of beads used ((i) 40 nm, (ii) 60 nm, (iii) 100 nm, and (iv) 200 nm).

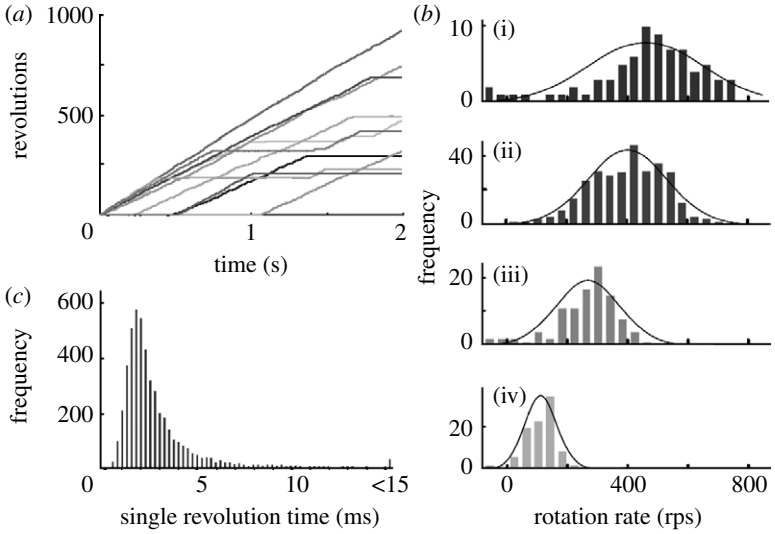

Figure 4. Stochastic fluctuation of the γ subunit rotation. (*a*) Time courses for the gold beads. Rotation of randomly selected beads (60 nm diameter) was followed. (*b*) Histograms of rotation rates. The rotation rates of beads were estimated every 10 ms ((i) 40 nm, (ii) 60 nm, (iii) 100 nm, and (iv) 200 nm). (*c*) *Single revolution time* of gold beads. The time course of 60 nm beads was followed, and *single revolution time* (time required for the 360° revolution) is shown as histogram. Histograms obtained for 10 randomly selected beads are shown (see Ref. Nakanishi-Matsui *et al.* 2007 for coloured version).

(Yasuda *et al.* 2001) or a fluorophore (Adachi *et al.* 2000). 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).

(b) *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 F_1 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 4*a*). Histograms of the rotation speed indicated that the rotation exhibited stochastic fluctuation (figure 4*b*) 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 dwells were 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 4*c*). 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 3*b*) 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 F_0F_1 (in preparation). These results indicate that the fluctuation is an intrinsic property of F-ATPase.

The effect of the ε subunit, an inhibitor of F_1 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 F_1 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 5*a*), 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,

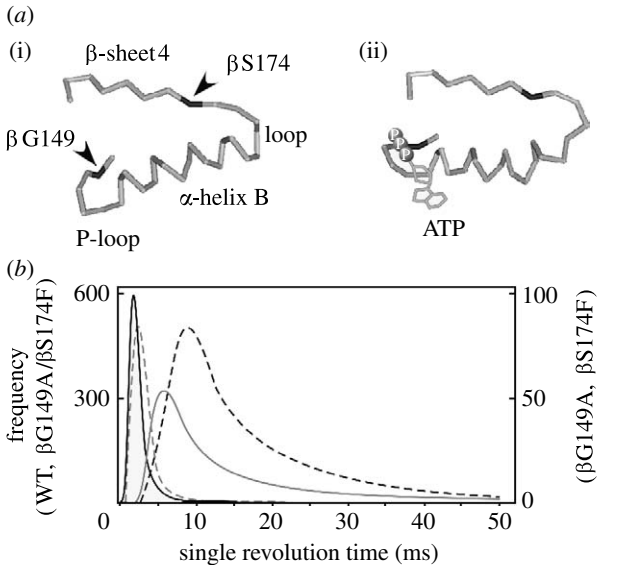

Figure 5. β subunit domain essential for F_1 rotation. (*a*) β Subunit domain between β-sheet4 and P-loop. A β subunit domain is shown for an (i) empty (β_E) β subunit or (ii) ATP bound (β_{TP}). The positions of Ser174, Gly149 and ATP are shown according to the bovine F_1 (Abrahams et al. 1994). (*b*) 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 F_1 (wild-type, black solid curve; Phe174, grey solid curve; Phe174/Ala149, grey dashed curve; and Ala149, black dashed curve).

suggesting that actin is not suitable for studying mutant F_1 (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 5*b*), 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 F_1 generated essentially the same torque regardless of the mutation (Omote *et al.* 1999), suggesting that the mutant γMet23Lys 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 *c*Asp61 or *a*Arg210 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

for each step may also be stochastic. As discussed above, F_1 has three catalytic sites, whereas proton transporting aspartate or glutamate in F_0 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 F_1. It should be noted that kinetic parameters determined on bulk phase measurement for ATPase are the averages for individual molecules.

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