Journal of Bioenergetics and Biomembranes, Vol. 32, No. 5, 2000

A Biological Molecular Motor, Proton-Translocating ATP Synthase: Multidisciplinary Approach for a Unique Membrane Enzyme

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Proton-translocating ATP synthase (F_oF_1) synthesizes ATP from ADP and phosphate, coupled with an electrochemical proton gradient across the biological membrane. It has been established that the rotation of a subunit assembly is an essential feature of the enzyme mechanism and that F_oF_1 can be regarded as a molecular motor. Thus, experimentally, in the reverse direction (ATP hydrolysis), the chemical reaction drives the rotation of a $\gamma\varepsilon c_{10-14}$ subunit assembly followed by proton translocation. We discuss our very recent results regarding subunit rotation in *Escherichia coli* F_oF_1 with a combined biophysical and mutational approach.

KEY WORDS: *Escherichia coli*; motor; ATP synthase; F_oF_1; catalytic residues; ion pump; ATPase; mechanochemistry; *c*-subunit ring; energy conversion.

INTRODUCTION

An electrochemical proton gradient across the mitochondrial, chloroplast, or bacterial membrane drives ATP synthesis from ADP and phosphate (P_i) by ATP synthase (also known as F_oF_1 or F_oF_1-ATPase) (see Futai *et al.*, 1989; Futai and Omote, 1996; Nakamoto, 1996; Boyer, 1997, for reviews). The enzyme is composed of two functional units, a transmembrane F_o sector and a membrane extrinsic F_1 sector. F_o has three types of subunits, *a*, *b*, and *c*, with a stoichiometry of 1:2:10–14, and forms a proton translocation pathway. F_1 ($\alpha_3\beta_3\gamma\delta\varepsilon$) is a catalytic sector formed from five different subunits, α, β, γ, δ, and ε (Fig. 1). The three β subunits have catalytic sites for ATP synthesis and hydrolysis. The isolated F_1 sector can not synthesize, but can hydrolyze ATP (F_1-ATPase).

The mechanism of ATP synthesis by F_oF_1 comprises three steps: (1) Proton translocation through F_o, (2) conformation transmission to F_1, and (3) ATP synthesis in the β subunit. Boyer proposed that the ATP synthesis occurs through successive conformational changes in the three catalytic β subunits; "tight" (β_T), ATP-bound; "loose" (β_D), ADP/P_i bound; and "open" or "empty" (β_E), no nucleotide (see Boyer, 1997, for a review). His hypothesis, the binding-change mechanism, also proposes that proton flux causes 120° rotation of the γ subunit relative to the $\alpha_3\beta_3$ hexamer, leading to conformational changes in the catalytic subunits. This mechanism is also called *rotational catalysis.*

Consistent with this mechanism, determination of the X-ray structure of bovine F_1 (Abrahams *et al.*, 1994) showed that three α and β subunits are arranged alternately around the γ subunit and that the β subunits have three states (β_T, β_D, and β_E, respectively). Furthermore, the relative orientation of the γ subunit, as to the three β subunits, is different. The kinetic model and the X-ray structure prompted studies to show the rotation of the γ subunit in F_1. Biochemical experiments provided the initial evidence for γ-subunit rotation (Kandpal and Boyer, 1987). Continuous rotation of a fluorescently labeled actin filament connected to a thermophilic *Bacillus* γ subunit was video recorded directly using an immobilized $\alpha_3\beta_3\gamma$ assembly (Noji *et al.*, 1997). A similar system for observing the rotation of the γ subunit in a single *E. coli* F_1 has also been

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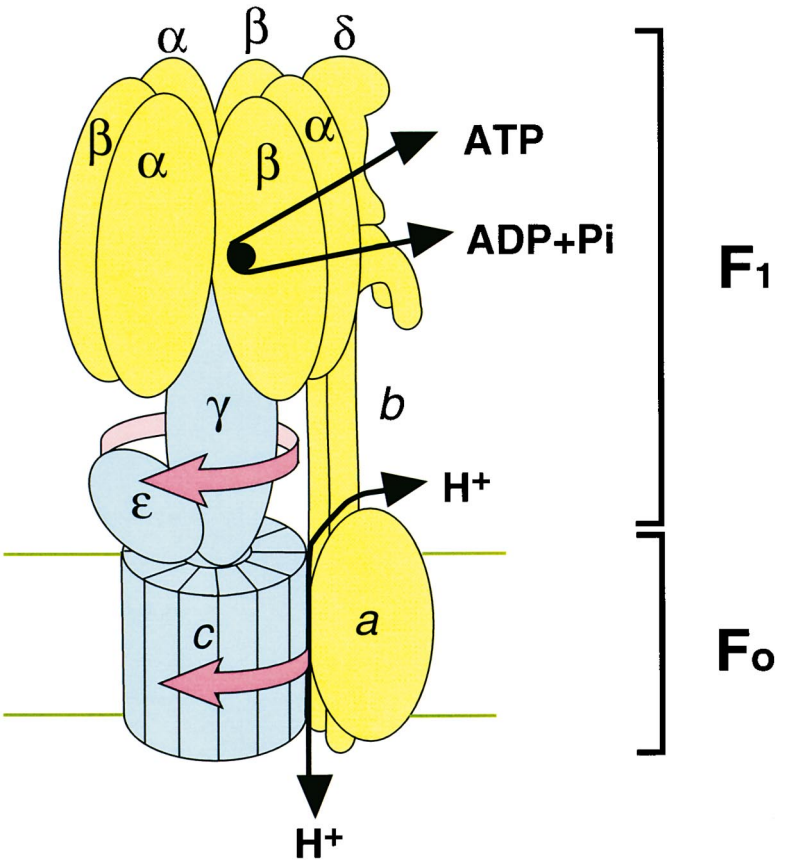

Fig. 1. Subunit organization of ATP synthase (F_oF_1). The rotor complex (white) and stator (grey) are shown schematically. We discuss in the text that these parts are interchangeable.

established (Omote *et al.*, 1999; Noji *et al.*, 1999). Progress in these areas have enabled us to analyze rotational catalysis, taking advantage of the wealth of information obtained through genetic and biochemical approaches. The next major issue was the observation of *c*-subunit rotation during ATP hydrolysis in F_oF_1 immobilized on a glass surface (Sambongi *et al.*, 1999; Pänke *et al.*, 2000).

In this article, we discuss the catalytic site in the F_1 sector and rotation of the subunit assembly in F_oF_1, emphasizing our own results for the *E. coli* enzyme. A multidisciplinary approach involving genetics, biochemistry, and biophysics is suitable for studying membrane enzymes.

β SUBUNIT CATALYTIC SITE AND REQUIREMENT OF γ FOR ENERGY COUPLING

Key Amino Acid Residues for Catalysis

Key amino acid residues identified in genetic or biochemical studies have often been confirmed by the X-ray structure. Moreover, random mutagenesis studies have revealed functionally important residues, which are difficult to identify only from the structure. Similarly, residues responsible for the basic chemistry in the catalytic site of F_oF_1 were identified in extensive mutagenesis studies on the *E. coli* enzyme (Futai and Omote, 1996) before the structural information became available. Indeed, *E. coli* F_oF_1 represents one of the best examples of the structure–function relationship studied by means of the combined approach of biochemistry, genetics, and structural biology.

Among residues identified in combined biochemical and genetic studies (Fig. 2), βLys155 and βThr156 (*E. coli* numbering) are in the β subunit glycine-rich sequence or P-loop (phosphate-binding loop, Gly-Ala-Gly-Val-Gly-Lys-Thr in β, so far sequenced) conserved among nucleotide binding proteins. The results of kinetic studies on the purified mutant F_1 (Hsu *et al.*, 1987; Takeyama *et al.*, 1990; Omote *et al.*, 1992; Senior and Al-Shawi, 1992) and affinity labeling of the wild-type F_1 with ATP analogs (Chuan and Wang, 1988; Ida *et al.*, 1991) suggested that the ε amino group of βLys155 interacts with

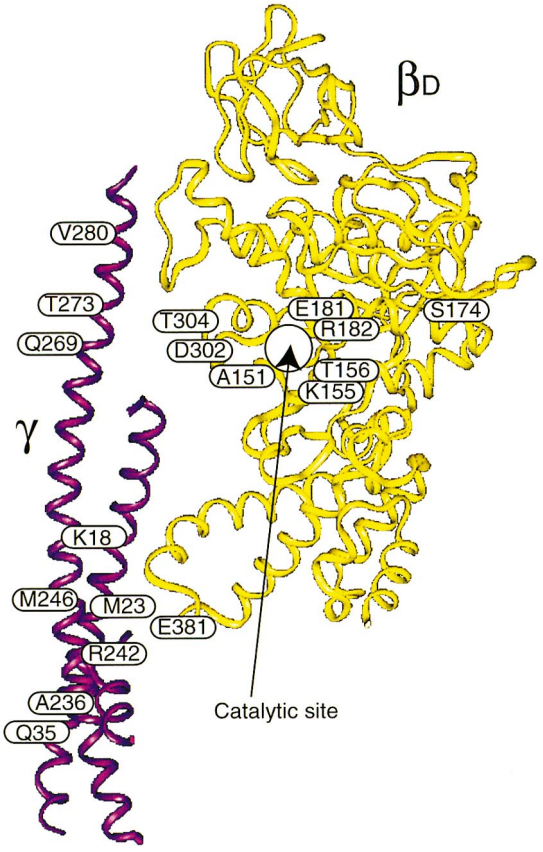

Fig. 2. The X-ray structure of the β and γ subunits in the $\alpha_3\beta_3\gamma$ complex. The structure of $\alpha_3\beta_3\gamma$ is cited from Abrahams *et al.* (1994). The catalytic site and amino acid residues discussed in this paper are indicated.

the β/γ phosphate moiety of the bound ATP. βThr156 could be substituted with serine without loss of activity, but not with other residues (Omote *et al.*, 1992), indicating that the hydroxyl moiety at position 156 contributes to Mg^{2+} binding in the catalytic site. As expected from these results, the X-ray structure of bovine F_1 revealed that βLys155 and βThr156 are close to ATP β/γ phosphate and Mg, respectively (Abrahams *et al.*, 1994). Replacement of a residue in the P-loop, such as βAla151, changed the ATPase activity level from 10 to 200% of the wild–type (Hsu *et al.*, 1987; Takeyama *et al.*, 1990). Thus, this residue is not directly involved in the chemistry, but contributes to maintenance of the conformation of βLys155 and/or βThr156.

The sequence of βGly180–βGlu185 (Gly-Glu-Arg-Arg-Thr-Glu in F_1 β) is also conserved among the β subunits of the various species (Futai and Omote, 1999). From the results of kinetic studies on mutant enzymes, βGlu181 and βArg182 are suggested to be catalytic residues (Senior and Al-Shawi, 1992; Park *et al.*, 1994). In the X-ray structure, the βGlu181 side chain forms a hydrogen bond with a water molecule located close to the γ-phosphate moiety of ATP (Fig. 2; Abrahams *et al.*, 1994). Therefore, βGlu181 should activate the water molecule during the hydrolysis of ATP.

The γ Subunit is Required for Catalysis, Energy Coupling, and Assembly

The amino and carboxyl terminal α helices of the γ subunit are located in the central space of the $\alpha_3\beta_3$ assembly (Fig. 1; Abrahams *et al.*, 1994). The results of biochemical studies, combined with extensive mutagenesis, suggested that the γ subunit is required for $\alpha_3\beta_3\gamma$ assembly, catalysis, and energy coupling (see Nakamoto *et al.*, 1992, for a review). The γMet23 to Arg or Lys (γM23K) mutation (Fig. 2) caused impaired ATP-dependent proton transport (Shin *et al.*, 1992) and ATP synthesis activities (Al-Shawi *et al.*, 1997a), but was less effective on ATP hydrolysis activity, indicating that the mutant is less efficiently coupled. Lys23 in the γM23K mutant is believed to form an ionized hydrogen bond with βGlu381 of the DELSEED loop (βAsp380–βAsp386) of the β_D subunit (Al-Shawi *et al.*, 1997b). Furthermore, the βGlu381 to Gln, Ala, or Asp mutation restored efficient coupling when expressed with γM23K (Ketchum *et al.*, 1998). Interestingly, a single mutation (βE381K) in the DELSEED loop also caused inefficient coupling. These results suggest that DELSEED and its interaction with the γ subunit are involved in energy coupling (Ketchum *et al.*, 1998). The X-ray structure also indicated that three β subunits interact differently with γ (Abrahams *et al.*, 1994).

The defect of γM23K was suppressed by a series of second-site mutations mapped between γGln269 and γVal280 (Nakamoto *et al.*, 1993), which do not interact directly with γMet23. γGln269 forms a hydrogen bond with each of βAsp302 and βThr304 in the β_E subunit loop between βAsp301 and βPro306 (Asp-Asp-Leu-Thr-Asp-Pro), as revealed by the X-ray structure (Abrahams *et al.*, 1994).

The γGln269→Glu or γThr273→Val substitution also severely affected ATPase activity and energy coupling (Fig. 2; Nakamoto *et al.*, 1995). The second-site mutations that suppress these mutations have been mapped to the amino (γ18–γ35) and carboxyl (γ236–γ246) terminal regions (Nakamoto *et al.*, 1995). Similar to the suppression of γM23K, the residues of primary (γ269 or γ273) and second-site (γ18–γ35 and γ236–γ246) mutations are not located nearby.

These findings suggest that the two α helices of the γ subunit undergo long-range conformation transmission.

Such transmission is an integral part of the energy coupling and catalytic mechanism and important for the successive interactions of the three catalytic β subunits with the two α helices.

ATP HYDROLYSIS DRIVES γ-SUBUNIT ROTATION IN F_1

From the $\alpha_3\beta_3\gamma$ Structure to γ-Subunit Rotation

The conformational differences between the three β subunit catalytic sites and their relative orientation as to the γ subunit are consistent with the rotation included in the binding change mechanism. As discussed above for γM23K, γ plays an important role by linking the proton transport (through F_o) to the chemistry in the catalytic subunits of F_1. The X-ray structure of F_1 prompted studies showing the γ rotation. These included cryoelectron microscopy of gold-labeled F_1 (Gogol *et al.*, 1990), β/γ subunit cross linking of βCys380 substituted in the DELSEED loop and endogenous γCys87 (Duncan *et al.*, 1995; Zhou *et al.*, 1997), and polarized absorption recovery after photobleaching (Sabbert *et al.*, 1996). The three-stepped rotation in a single F_1 was shown by confocal fluorometry of a probe attached to the γ or ε subunit (Häsler *et al.*, 1998). The chemical cross-linking approach further demonstrated rotation of the ε subunit relative to β in membrane-bound F_oF_1 (Zhou *et al.*, 1996).

The continuous unidirectional γ-subunit rotation in F_1 was observed directly by Noji *et al.* (1997). The thermophilic *Bacillus* $\alpha_3\beta_3\gamma$ assembly was immobilized on a Ni-coated glass surface through histidine tags (ten histidine residues) introduced at the β subunit amino termini and a fluorescently labeled actin filament was connected to the γ subunit. Rotation of the filament was observed upon ATP addition. The filaments could rotate anticlockwise and exhibited a 120° step consuming a single molecule of ATP (Yasuda *et al.*, 1998). This pioneering experiment clearly indicated that the chemistry at the catalytic site is coupled with the mechanical work in the isolated F_1.

Frictional torque of ~40 pN·nm was generated on the γ-subunit rotation, as calculated from the viscous drag and rotational rate of the filaments (Yasuda *et al.*, 1998). Therefore, the energy required for 120° rotation of γ is ~84 pN·nm (=40 pN·nm $\times$ $2\pi/3$), which is nearly equal to the Gibbs free energy change of the hydrolysis of a single ATP under physiological conditions [(~12 kcal/mol)/6.0×10^{23} = ~83 pN·nm]. These results indicate that the efficiency of energy conversion between the chemistry and γ rotation is nearly 100%. Kato-Yamada *et al.* (1998) also showed the rotation of an actin filament connected to the ε subunit, indicating that the $\varepsilon\gamma$ assembly is a rotor in isolated F_1.

Escherichia coli γ-Subunit Rotation

We have also established an experimental system for the direct observation of the γ rotation in *E. coli* F_1 (Omote *et al.*, 1999). α or β subunits were fixed on a glass surface through histidine tags at the amino termini and, then, a fluorescently labeled actin filament was connected to the γ subunit through streptavidin. The anticlockwise rotation of the filament could be observed on the addition of ATP. The *E. coli* γ subunit generated essentially the same frictional torque (Omote *et al.*, 1999) as that of the *Bacillus* (Yasuda *et al.*, 1998). The filament stopped rotating immediately after the addition of sodium azide, a potent inhibitor of F_1-ATPase, supporting the idea that the rotation is dependent on the ATP hydrolysis by F_1. Noji *et al.* (1999) also observed rotation of the *E. coli* γ subunit. Thus, it became possible to analyze the F_1 rotational catalysis in detail, taking advantage of the extensive information on the *E. coli* enzyme obtained through genetic and biochemical analysis.

γM23K (γMet23 replaced by Lys) is the first mutant, which we have analyzed as to the rotational catalysis (Omote *et al.*, 1999). Introduction of the γM23K mutation into F_oF_1 engineered for rotation gave similar phenotypes to those of the original mutant: the engineered F_oF_1 (γM23K) exhibited essentially the same ATPase activity but reduced ATP-dependent proton translocation. The mutant γ generated essentially the same torque as the wild type, indicating that the γM23K enzyme is defective in the coupling at the interface between F_1 and F_o. However, it may be possible that the difference between the wild-type and γM23K rotation could not be determined due to the scatter of the experimental points. Thus, the deviation of the rotational rates should be carefully evaluated for a final conclusion. It is necessary to determine whether the method for analyzing the rates and torque generation can be improved.

An obvious question is how ATPase activity is related to the γ-subunit rotation. When the scales for the rotation and time were expanded, we observed that the rotational rate varied slightly during the video recording. Furthermore, rotation of the filaments often ceased and started again on a subsecond scale in the presence of ATP (mM range). We only studied filaments well separated from each other. Thus, interaction between different filaments was excluded. Therefore, we could observe intrinsic pauses on comparison of the mutant and wild-type rotation under the same conditions, including the length of filaments.

With this careful consideration, we could determine the rotational rate (dependent on the viscous drag against the filaments) and pausing frequency. Interesting mutants for analysis are those involving substitution of βSer174 and βAla151; βSer174 is located in the hinge loop of the catalytic domain and Gly, Ala, Thr, and Leu (or Phe) substitution gave 150, 120, 70, and 10% of the wild-type ATPase activity, respectively (Omote *et al.*, 1994). βAla151 is in the P-loop and Val and Pro substitution gave 10 and 200% of the ATPase activity, respectively (Hsu *et al.*, 1987; Takeyama *et al.*, 1990). We have already found that the γ-subunit rotation in the βS174F mutant showed a higher frequency of pauses with 120° intervals (Iko *et al.*, unpublished observation).

ROTATIONAL CATALYSIS IN THE F_OF_1 HOLO ENZYME

c Subunit Ring and Its Rotation

The γ- and ε-subunit rotation in F_oF_1 during ATP synthesis was shown by a cross-linking experiment (Schulenberg *et al.*, 1999). Thus, it is beyond doubt that the subunit rotation should occur during ATP synthesis and hydrolysis in the entire F_oF_1. One of the remaining important questions is how the proton translocation through F_o drives the rotation of the $\gamma\varepsilon$ assembly during ATP synthesis or, conversely, how ATP hydrolysis-dependent γ rotation is transmitted to the F_o sector to drive proton transport across the membrane. It is not easy to answer these questions. However, mechanical coupling between F_1 and F_o and *c*-subunit rotation have been proposed (Junge *et al.*, 1997; Elston *et al.*, 1998) and experimentally supported (Sambongi *et al.*, 1999; Pänke *et al.*, 2000).

Studies involving electron and atomic force microscopy have indicated that *c* subunits form a ring (Brikenhäger *et al.*, 1995; Takeyasu *et al.*, 1996; Singh *et al.*, 1996). Dmitriev *et al.* (1999), and Rastogi and Girvin (1999) proposed a ring comprising 12 monomer NMR structures of the *E. coli c* subunit. Determination of the X-ray structure of the yeast F_1 *c* subunit oligomer showed that 10 copies of *c* form an almost symmetrical ring (Stock *et al.*, 1999). Atomic force microscopy of chloroplast F_o showed a symmetric ring of 14 *c* subunits (Seelert *et al.*, 2000). Thus, it is of interest to know how the *c* ring functionally couples with the $\gamma\varepsilon$ rotation.

c Subunit ring rotation with γ has been proposed for the coupling between the F_1 and F_o sectors (Junge *et al.*, 1997; Elston *et al.*, 1998). Consistent with this model, the chemical cross linking between γ and *c*, similar to that between γ and ε, did not affect ATP hydrolysis-dependent proton transport (Watts and Capaldi, 1997; Ketchum and Nakamoto, 1998), whereas the cross linking of α or β with γ resulted in loss of activity (Aggeler *et al.*, 1995). It is obvious that the cross linking within the rotor (or stator) subunits does not affect rotation, but that between the stator and rotor inhibits it completely. The X-ray structure for the yeast enzyme indicates that γ, ε, and *c* subunit ring (10 *c*) are tightly associated (Stock *et al.*, 1999), supporting the $\gamma\varepsilon c$ rotation as an ensemble relative to the $\alpha_3\beta_3$ assembly.

Rotation of An Actin Filament Connected to the *c* Ring

We provided the first direct evidence of continuous *c*-ring rotation during ATP hydrolysis (Sambongi *et al.*, 1999). *Escherichia coli* F_oF_1 was immobilized on a glass surface through histidine tags introduced into the α subunits and fluorescently labeled actin filaments were attached to the genetically introduced cysteine residues (*c*Glu2→Cys) of the *c* subunits via biotin–maleimide chemical modification and streptavidin (Fig. 3). We could observe filament rotation after the addition of ATP. A series of control experiments confirmed that a rotating filament was attached to the *c* subunit. We also purified the F_1 sector from the same strain as that examined for the *c*-ring rotation in F_oF_1: actin filament binding through biotin–maleimide modification to an engineered γ subunit (Cys-less) and its rotation were not detectable under our conditions (Sambongi *et al.*, 1999). The direction of filament rotation and the torque generated for the *c* ring were the same as those for a filament connected to the γ subunit (γCys193) in F_1. The rotation was inhibited by an F_oF_1-ATPase inhibitor, venturicidin (Fillingame *et al.*, 1991), whereas that of the *c*I28T (*c*Ile28→Thr) mutant was not (Sambongi *et al.*, 1999, and Tanabe *et al.*, unpublished observation). Our results and those of structural analyses indicated that the *c* subunit ring rotates together with the γ and ε subunits in F_oF_1 during ATP hydrolysis and synthesis.

Pänke *et al.* (2000) more recently also observed *c* subunit rotation during ATP hydrolysis using the improved system. F_oF_1 was immobilized through histidine tags introduced into the β subunits. They genetically introduced a "strep tag" peptide sequence into the *c* subunit; the tag was used specifically to attach a fluorescently labeled actin filament via streptactin. The rotational characteristics were the same as those with our system (Sambongi *et al.*, 1999).

Tsunoda *et al.* (2000) claimed that they could not connect an actin filament to a *c* ring under their conditions and raised two problems with *c* ring rotation: (1) the

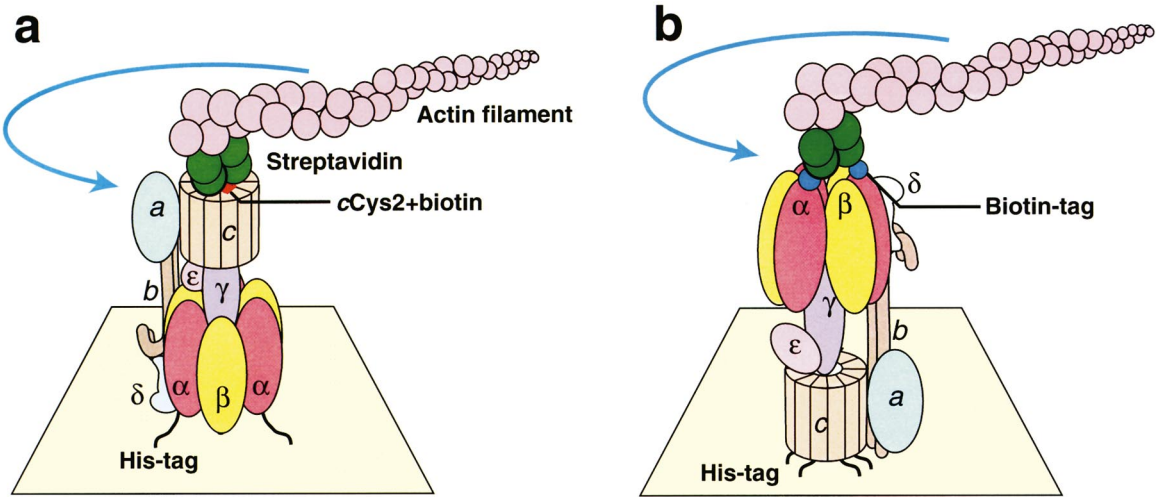

Fig. 3. Experimental system for observing rotation in F_oF_1. The systems used for subunit rotation are schematically shown. F_oF_1 was immobilized through a histidine tag attached to the α (a) or *c* (b) subunit and a fluorescent actin filament was connected to the *c* (a) or α (b) subunit. A similar system for introducing a histidine tag or actin filament to the β subunit was also examined (unpublished).

actin filament connected to the γ subunit (Cys-less) of F_1, possibly contaminating F_oF_1, rotated instead of the *c* ring; and (2) F_oF_1 in which the rotation was observed may not have all subunits and they may be functionally uncoupled. Argument (1) was directed toward Sambongi *et al.* (1999), but is circumstantial. Tsunoda *et al.* (2000) assumed that biotin–maleimide reacted with the lysine ε amino moiety of the Cys-less γ of the F_1 sector contaminating the F_oF_1 preparation. There are only ten candidate residues for such modification, although their exact orientations are unknown and the reactivity of maleimide with the amino moiety is much lower than with thiol at neutral pH (Brewer and Riehm, 1967). The possibility that the lysine residues in γ to attach to an actin filament through maleimide is low, as discussed above.

As we carried out extensive control experiments (Sambongi *et al.*, 1999) and further results will be published elsewhere (Tanabe *et al.*, submitted; Sambongi *et al.*, submitted), we need not discuss this in more detail. However, two points should be made to avoid confusion among readers: Tsunoda *et al.* (2000) reacted F_oF_1 with fluorescein-5-maleimide and then biotinylated antifluorescein IgG and an actin filament through streptavidin; or with biotin–maleimide and then an actin filament through streptavidin. The first protocol was entirely different from that of Sambongi *et al.* (1999). The second protocol was similar to that of Sambongi *et al.* (1999), but the position of the *c* subunit cysteine residue was different: Tsunoda *et al.* (2000) inserted a cysteine between *c*Glu2 and *c*Asn3, whereas Sambongi *et al.* (1999) changed *c*Glu2 to Cys. Thus, the orientations of Cys introduced in the two experiments were not the same. The conditions for modification of F_oF_1 with a maleimide derivative were different: *i.e.*, Tsunoda *et al.* (2000), pH 7.5, 25°C for 20 min and Sambongi *et al.* (1999), pH 7.0, 4°C for 60 min. Other conditions were also different between the two groups, although the differences may be minor. Therefore, the arguments of Tsunoda *et al.* (2000) were based on different experiments. It is unfortunate that their criticisms were made without extensive discussion between the two groups.

The second criticism (the argument 2) has some significance and can be directed toward Pänke *et al.* (2000) and Sambongi *et al.* (1999). It should be noted that the two groups did not definitely conclude that the actual rotating molecule has all the subunits. As discussed by Pänke *et al.* (2000), it is difficult to prove that all the subunits are integrated into the rotating enzyme. The scientific importance of the two publications is that they show that the *c* subunit ring was rotating when F_oF_1 was immobilized. It is without doubt that the rotating $\gamma\varepsilon c$ should be included in the mechanism of the F_oF_1 holo enzyme as the γ-subunit rotation in the immobilized $\alpha_3\beta_3\gamma$ could be.

The Rotor and Stator Are Interchangeable

We further examined whether the rotor and stator in F_oF_1 are interchangeable. The actin filament was specifically connected to the α subunits using streptactin or a biotin tag and the *c* subunits were fixed on a glass surface (Fig. 3). If the *c* subunit ring is immobilized, *c*, γ, and

ε form a stator, and $\alpha_3\beta_3ab_2$ should rotate. The filament connected to the α subunits could rotate using the energy derived from ATP hydrolysis; the rotational direction and rates were as expected from the results for the *c* ring rotation (Sambongi *et al.*, unpublished observation). This experiment clearly indicated that the internal rotation in the F_oF_1 holo enzyme is relative. The *c* ring rotation relative to the $\alpha_3\beta_3$ complex ruled out the model in which *c* subunit conformational changes force the γ and ε subunits to move along the top of the *c* ring in a circular direction (for example, discussed in Rastogi and Girvin, 1999).

A BIOLOGICAL MOLECULAR MOTOR, F_OF_1

As discussed in this article, ATP synthase is a unique enzyme, coupling chemistry (ATP synthesis or hydrolysis), subunit rotation, and proton translocation. It has been established that rotation of the $\varepsilon\gamma c$ assembly relative to $\alpha_3\beta_3ab_2$ is an essential feature of the energy coupling between proton translocation through F_o and ATP synthesis or hydrolysis in F_1. Studies on ATP hydrolysis-dependent subunit complex rotation together with various mutations will facilitate establishment of the molecular mechanism of the enzyme.

One of the most important experiments required for further understanding is one showing subunit complex rotation coupled with ATP synthesis from ADP and P_i when an electrochemical gradient is applied. The direction of the rotation during ATP synthesis may be opposite to that during ATP hydrolysis. However, such an experimental system may not be easy to establish. In this regard, F_oF_1 immobilized through the *c* ring may be easily incorporated into a phospholipid bilayer sheet. A further exciting experiment will be one showing ATP synthesis when the γ subunit in immobilized F_1 is rotated artificially at different rates. Such experiments will correlate the physical parameters of rotation to the chemistry of ATP synthesis/hydrolysis.

ACKNOWLEDGMENTS

The authors are grateful to the co-workers whose names appear in the references.

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