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Observations of rotation within the F_oF_1-ATP synthase: deciding between rotation of the F_oc subunit ring and artifact

Satoshi P. Tsunoda[a], Robert Aggeler[b], Hiroyuki Noji[c], Kazuhiko Kinosita Jr.[c,d], Masasuke Yoshida[a,c,*], Roderick A. Capaldi[b]

[a] *Research Laboratory of Resources Utilization, Tokyo Institute of Technology, 4259 Nagatsuta, Yokohama 226-8503, Japan*
[b] *Institute of Molecular Biology, University of Oregon, Eugene, OR 97403-1229, USA*
[c] *CREST (Core Research for Evolutional Science and Technology) Genetic Programming Team 13, Teikyo University Biotechnology Research Center 3F, 907 Nogawa, Miyamae, Kawasaki 216-0001, Japan*
[d] *Department of Physics, Faculty of Science and Technology, Keio University, 3-14-1, Hiyoshi, Yokohama 223-8522, Japan*

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Abstract **F_oF_1-ATP synthase mediates coupling of proton flow in F_o and ATP synthesis/hydrolysis in F_1 through rotation of central rotor subunits. A ring structure of F_oc subunits is widely believed to be a part of the rotor. Using an attached actin filament as a probe, we have observed the rotation of the F_oc subunit ring in detergent-solubilized F_oF_1-ATP synthase purified from *Escherichia coli*. Similar studies have been performed and reported recently [Sambongi et al. (1999) Science 286, 1722–1724]. However, in our hands this rotation has been observed only for the preparations which show poor sensitivity to dicyclohexylcarbodiimde, an F_o inhibitor. We have found that detergents which adequately disperse the enzyme for the rotation assay also tend to transform F_oF_1-ATP synthase into an F_o inhibitor-insensitive state in which F_1 can hydrolyze ATP regardless of the state of the F_o. Our results raise the important issue of whether rotation of the F_oc ring in isolated F_oF_1-ATP synthase can be demonstrated unequivocally with the approach adopted here and also used by Sambongi et al.**

Key words: F_oF_1-ATP synthase; Rotation; *c* subunit ring; Detergent; Uncoupling

1. Introduction

The F_oF_1-ATP synthase (F_oF_1) is a ubiquitous, important enzyme, which couples proton flow and ATP hydrolysis/synthesis: it catalyzes ATP synthesis by utilizing the energy of downhill proton flow across membranes and as a reverse reaction it can pump protons by ATP hydrolysis. This enzyme is composed of two moieties, F_o and F_1. The F_o moiety ($ab_2c_{10-12?}$ in bacterial F_oF_1) is embedded in the membrane and mediates proton translocation. The F_1 moiety ($\alpha_3\beta_3\gamma\delta\varepsilon$) protrudes from the membrane, has the catalytic center of the ATP synthesis/hydrolysis and is easily detached from F_o in a reversible manner. Isolated F_1 retains ATP hydrolysis activity but not ATP synthesis function. A central unresolved issue is the mechanism of the coupling reaction, an energy exchange between the ion flow through F_o and ATP synthesis/hydrolysis in F_1 [1]. The rotary mechanism proposed independently by Boyer [2], Oosawa [3] and Cox et al. [4] predicted that the energy would be exchanged through rotation of the rotor subunit(s). This general idea was supported by the crystal structure of bovine heart mitochondrial F_1 [5]. Indirect evidence of the large movements of the central γ in the $\alpha_3\beta_3$ ring during catalysis was obtained in several studies [6–9]. Convincing evidence for this concept came with a direct visualization of rotation of actin filament attached to γ in F_1's from a thermophilic *Bacillus* PS3 [10,11], *Escherichia coli* [12,13], and spinach chloroplast [14]. Also the rotation of ε subunit has been demonstrated [15].

The obvious next question then is how ion flow through the F_o sector generates the rotation of γ. It is now widely believed that the ring of F_oc constitutes a rotor assembly with γ [16–18], supported by the observations that the F_oc ring has a direct contact with the γ and ε [19,20] and that the cross-linking of F_oc and γ does not impair ATPase activity of F_oF_1 [21]. However, more definitive evidence requires direct observation as for the γ subunit. To this end we have bound the actin filament to the F_oc of *E. coli* F_oF_1. Rotation of the actin filament in response to ATP has been observed. Unexpectedly, however, dicyclohexylcarbodiimide (DCCD), an inhibitor specific to F_o, did not inhibit this rotation. We have found that gel filtration in the presence of detergent is responsible for the loss of DCCD sensitivity indicating that detergent at levels needed for dispersing F_oF_1 disrupts the complex. Meanwhile the rotation of the F_oc ring has been reported very recently by Sambongi et al. employing essentially the same techniques and material [22]. Their report has been taken as a final proof of rotation of the F_oc ring [23]. We present results that focus attention on the artifacts that can limit interpretation of such single particle studies.

2. Materials and methods

2.1. Strains, plasmids and isolation of F_oF_1

M13mp18 containing the 1.2-kb *Fsp*I/*Sac*I fragment with the N-terminus of *unc*D was used for site-directed mutagenesis to introduce a His-tag at the N-terminus of the β subunit with the oligonucleotide GGATTTAAGATG*(CATCAC)*$_5$GCTACTGGAAAG (italic, inserted base) according to Kunkel et al. [24]. The 933-bp *Stu*I/*Sac*I fragment was inserted in pRA100, creating pRA183. M13mp18 containing the 2.7-kb *Hin*dIII/*Sfi*I fragment of pRA100 in *Hin*dIII/*Sma*I

*Corresponding author. Fax: (81)-45-924 5277.
E-mail: myoshida@res.titech.ac.jp

Abbreviations: BM, biotin-PEAC5-maleimide; FM, fluorescein-5-maleimide; DCCD, dicyclohexylcarbodiimide

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was used for insertion of a cysteine residue between Glu2 and Asn3 of the F_oc subunit encoding *unc*E with the oligonucleotide GGAGACTGTCATGGAA*TGC*AACCTGAATATGGATC. The 1.6-kb *Dra*III fragment of this plasmid was then ligated with the 5-kb and 6-kb *Dra*III fragments of pRA183 to generate pRA186, which contains a His-tag at the N-terminus of β and a cysteine at the N-terminus of F_oc. An alternative His-tag was introduced at the N-terminus of the β subunit by replacing the 0.9-kb *Rsr*II/*Sac*I fragment of pRA186 with the one of pSK11 (a generous gift of Dr. Holger Lill, Universität Osnabrück), generating pRA190. A mutant with a cysteine-free γ subunit was constructed by introducing the γC112S mutation into the M13mp18 which contained a serine instead of cysteine in position 87 of the γ subunit [25] using the oligonucleotide AAAGGCGTTCAA*TCG*GACCTCGCAATG. The 1.1-kb *Sfu*I/*Eco*RI fragment was inserted in pRA13 [9]. Then the 1.8-kb *Rsr*II/*Xho*I fragment from this plasmid was ligated with the 10.8-kb fragment of pRA186 to create pRA216, containing a His-tag in β, a cysteine residue at the N-terminus of c and no cysteines in γ. A mutant containing a D242V mutation in β and a cysteine in the F_oc subunit was created by placing the 1-kb *Nco*I fragment of pMKS2 (a generous gift of Dr. Alan Senior, University of Rochester, [26]) into pRA13 [9]. The 5.8-kb *Xho*I/*Nsi*I fragment of this construct was then inserted in pRA186, creating mutant pRA232. *E. coli* strains CJ236 (New England Biolabs, UK) and XL1 Blue (Stratagene, USA) were used for site-directed mutagenesis and cloning. RA1 (*unc*$^-$) [27] was used for transformation with *unc* operon containing mutant plasmids. F_oF_1 was isolated as described by Foster and Fillingame [28] and modified by Aggeler et al. [29] followed by gel filtration column (PD10, Amersham Pharmacia, Sweden) equilibrated with buffer A (50 mM 3(*N*-morpholino) propanesulfonic acid (MOPS)-NaOH, pH 7.5, 5 mM $MgCl_2$, 10% glycerol and 0.05% lysolecithin). Alternatively, His-tag containing ATP synthase mutants could be isolated with an Ni-NTA column essentially according to Schulenberg [30]. Inner membranes at 80 units/ml buffer B (25 mM Tris–HCl, pH 7.8, 10% glycerol, 5 mM *p*-aminobenzamidine and 2 mM Mg-ATP) were treated with 1 mM phenylmethylsulfonylfluoride, 300 mM KCl, 1.2% deoxycholate and 0.48% cholate, stirred on ice for 5–10 min and centrifuged at 100 000×*g* for 20 min. Ni-NTA agarose beads were added to the supernatant (1 ml/5 mg protein) in buffer B. At a detergent concentration of 0.4% and 10 mM imidazole the mixture was shaken for 1 h at 4°C. The beads were placed in a column and washed with 3 volumes buffer B containing 0.2% tauro-deoxycholate and 10 mM imidazole and then with 1 volume buffer B containing 0.05% lysolecithin and 10 mM imidazole. ATP synthase was eluted with buffer B containing 0.05% lysolecithin and 150 mM imidazole. All His-tagged mutants described in this study showed 70% ATP hydrolysis activity of wild-type. F_oc-dependent DCCD sensitivity and ATP-dependent proton translocation, as determined by ACMA (9-amino-6-chloro-2-methoxy acridin) quenching on inner membranes, were the same as for wild-type, indicating coupling of F_1 and F_o.

2.2. Rotation assay of F_oF_1

Purified *E. coli* F_oF_1 in buffer A was treated with a 1-to-10 molar ratio of biotin-PEAC5-maleimide (BM) (Dojindo, Japan) or fluorescein-5-maleimide (FM) (Molecular Probes, USA) for 20 min at 25°C. Unreacted maleimide was removed by passing the sample through a gel filtration column (PD10) equilibrated with buffer A. The FM-labeled F_oF_1 was reacted with an equimolar amount of anti-fluorescein IgG (Molecular Probes) for 20 min on ice. BM-labeled F_oF_1 was reacted with a 10-fold excess of streptavidin for 20 min on ice and then subjected to HPLC gel filtration (Superdex 200, Amersham Pharmacia) in buffer C (50 mM MOPS-NaOH, pH 7.5, 5 mM $MgCl_2$, 5% glycerol and 0.025% lysolecithin). The F_oF_1 fractions were collected and used for the rotation assay, which was carried out at 25°C as reported previously with minor modifications [10,11]. 0.1% of Ni-NTA beads in buffer D (50 mM MOPS-KOH, pH 7.0, 50 mM KCl, 5 mM $MgCl_2$) were infused into a flowcell (10 μl) and allowed to adhere to the glass surface for 5 min. The chamber was washed twice with 3 volumes of buffer E (buffer D plus 10 mg/ml bovine serum albumin). Infusion, incubation and washing steps were then performed as follows; 15–150 nM of IgG-F_oF_1 or streptavidin-F_oF_1 in buffer C (1 volume); 5 min incubation; washing twice with 3 volumes of buffer E; infusion of 400 nM streptavidin; 5 min incubation; washing twice with 3 volumes of buffer E (in the case of streptavidin-F_oF_1, these three steps were omitted); infusion of 100 nM labeled actin filament (1 volume); 15 min incubation; washing twice with three volumes of buffer E; infusion of 1 volume of buffer F (buffer E plus 0.5% 2-mercaptoethanol, 0.2 mg/ml glucose oxidase, 30 U/ml catalase, 6 mg/ml glucose and 5 mM Mg-ATP). The observation and analysis of the rotation were described previously [11].

2.3. Assay of ATP hydrolysis

ATPase activity was measured at 37°C in the presence of an ATP regenerating system. The assay mixture contained 50 mM MOPS-KOH (pH 7.0), 50 mM KCl, 2.5 mM phospho*enol*pyruvate, 5 mM Mg-ATP, 0.2 mM NADH, 50 μg/ml pyruvate kinase and 50 μg/ml lactate dehydrogenase. If stated, detergent was added to the assay mixture. Inhibition by DCCD and venturicidin were measured as follows. DCCD was dissolved in ethanol at a concentration of 4 mM. F_oF_1 in buffer A containing 0.05% lysolecithin was incubated in the absence or in the presence DCCD (final concentration, 40 μM) for 60 min at 25°C. Ethanol concentrations were adjusted to 1% for all samples. An aliquot was placed in the ATPase assay mixture (50-fold dilution) containing the indicated detergent and the rate of ATP hydrolysis was measured. Lysolecithin and deoxycholate were obtained from Sigma (USA) and dodecylmaltoside and Triton X-100 were from Calbiochem (USA) and Nacalai (Japan), respectively. Venturicidin (a kind gift from Dr. Robert H. Fillingame, University of Wisconsin) was dissolved in dimethylsulfoxide. F_oF_1 in buffer A containing low concentration of lysolecithin (0.001%) was incubated in the assay mixture in the absence or presence of 70 μM venturicidin. All the samples contained 1% dimethylsulfoxide. After 2 min at 37°C, the indicated detergent was added to the assay mixture and the rate of ATP hydrolysis was measured. Since 1% dimethylsulfoxide had a small reducing effect on ATPase activity, the values without inhibitor were slightly smaller in the experiments with venturicidin than those in the experiments with DCCD.

3. Results

3.1. Observation of the rotation

For initial rotation studies an *E. coli* F_oF_1 mutant was generated with a His-tag introduced at the N-termini of the β subunits and with a cysteine inserted between Glu2 and Asn3 of F_oc and two intrinsic cysteines in γ replaced with serines. The intact enzyme was isolated as before and dispersed for measurements by using lysolecithin as a detergent. Other detergents were also tested (see later). Two procedures were used to attach an actin filament to F_oc; through fluorescein, anti-fluorescein IgG (biotinylated) and streptavidin (Fig. 1A) or through biotin and streptavidin (Fig. 1B). The F_oF_1 molecule was immobilized on a Ni-NTA coated solid

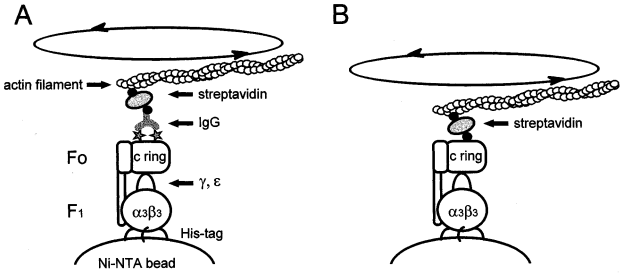

Fig. 1. Experimental setup for the observation of the F_oc ring rotation. F_oF_1 molecules from *E. coli* were immobilized through His-tags in the β subunits on the surface of Ni-NTA beads [11], which were adhered to a cover slip. The biotinylated actin filament, which was labeled with a fluorescent probe and F_oF_1 were connected through streptavidin, biotinylated anti-fluorescein IgG and fluorescein-5-maleimide (A) or through streptavidin and biotin-PEAC-maleimide (B) which was reacted with introduced Cys residues in F_oc. The star and black circle indicate fluorescence and biotin labels, respectively.

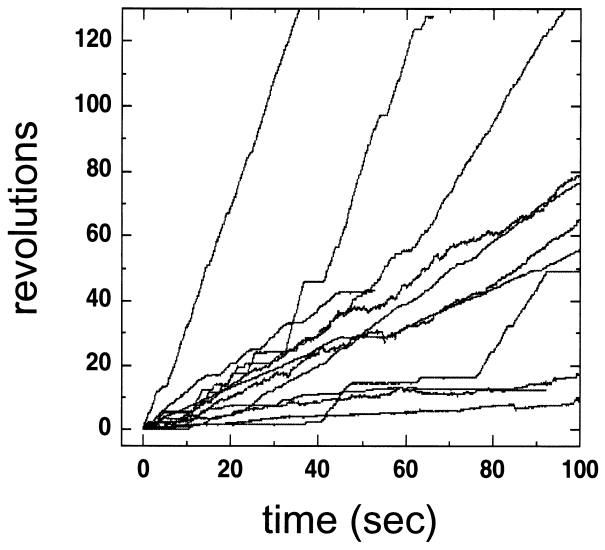

Fig. 2. Examples of time courses of the rotation of the actin filament presumably attached to the F_oc ring of F_oF_1.

surface through the His-tag. Detergent was removed from the reaction chamber after immobilization of F_oF_1 by repeated washing with the detergent-free buffers. A Mg-ATP-dependent rotation of the actin filament with counterclock-wise direction was observed for both FM-labeled and BM-labeled F_oF_1 (Fig. 2). The properties of the rotation of the actin filament including torque were very similar to those of the rotation of γ in F_1 [10]. However the frequency of finding rotating actin filaments was poor, roughly 0.12% of the total filaments. When we treated F_oF_1 with DCCD, the ATPase activity of F_oF_1 was inhibited by only a small amount (10–20%). This DCCD-treated F_oF_1 was immobilized and rotation was examined. The frequency of finding rotating filaments and the properties of the rotation were not significantly changed from those observed for the untreated F_oF_1. DCCD reacts covalently with Asp61 of F_oc [31], an essential residue for proton translocation and irreversibly inactivates the proton-translocation coupled ATP hydrolysis/synthesis activity of F_oF_1. The above results suggested two possible sources of artifact that needed to be considered. The observed rotation could be due to molecules in which γ rather than F_oc subunits had become labeled with actin and/or could reflect uncoupled enzyme that had been disrupted in a way that allows artifactual rotation of the F_oc subunit ring.

3.2. A critical test for rotation being due to actin binding in F_1

The rotation assay using an attached actin filament is highly powerful in finding rare rotating molecules. In particular it is natural to assume that F_oF_1 preparations contain free F_1 or F_1 loosely attached to F_o. If an actin filament attaches accidentally to γ of such an F_1, DCCD-insensitive rotation would be observed. We re-checked carefully if the labeling by BM and FM was exclusively restricted to the introduced cysteines in F_oc. Both for FM and BM labeling, only the F_oc band was stained by the usual protein immunoblotting method. However, when the blotted membrane was stained for considerably longer to identify modifications that occur at 1/100 or 1/1000 molecules, many undesired bands including γ appeared, whereas no band was stained in the control F_oF_1 without label (data not shown). This is not surprising because even though maleimides are highly specific for sulfhydryl groups, they can also react with amino groups as a minor side reaction. To avoid such undesired labeling, we replaced the F_1 moiety of FM-labeled F_oF_1 with a strip/reconstitution procedure [32] using new F_1 which had not been exposed to FM (Fig. 3). F_oF_1 used for the FM labeling step lacked the His-tag and was ATPase-inactive because of introduction of the mutation β-D242V [33]. Thus, even if F_1 derived from FM-labeled F_oF_1 should remain and re-assemble with F_o, this F_oF_1 would be unable to attach to the solid surface, beside being inactive as an ATPase. The F_1 used for the reconstitution contained His-tags at the N-termini of the β subunits and had wild-type ATPase activity. This procedure eliminates any possibility of ATPase-driven rotation of an actin filament accidentally attached to γ (or ε). The reconstituted F_oF_1 showed a full ATP dependent proton translocation on the inner membrane (measured by ACMA quenching), indicating the coupling of F_1 and F_o (data not shown). The rotation assay was carried out with this reconstituted F_oF_1, but no rotating actin filaments were found out of 12 000 actin filaments.

3.3. Effect of detergents on the function of F_oF_1

Prior to the rotation assay, labeled F_oF_1 was passed through a gel filtration column in the presence of a detergent (0.025% lysolecithin) to remove unbound IgG. Before gel filtration, with significant amounts of lipid present, the ATPase activity of F_oF_1 showed good sensitivity to DCCD inhibition (~80%). After gel filtration which can be expected to remove lipid as well as the IgG, it became almost insensitive (~10–20%). In one set of experiments we omitted the gel filtration step and looked for rotation. However, this DCCD-sensitive F_oF_1 did not show the rotation at all. This led us to examine

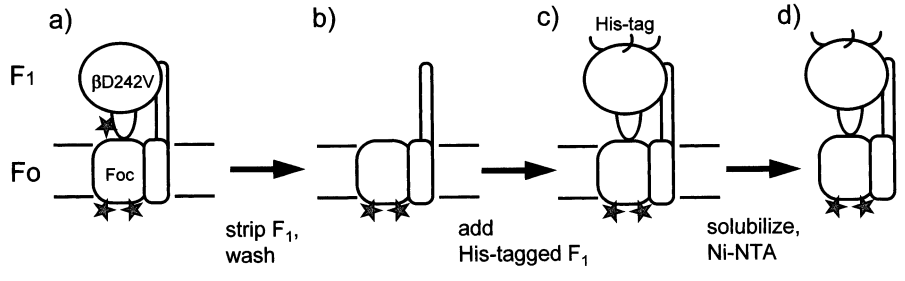

Fig. 3. Exchange of F_1 in F_oF_1 by the strip/reconstitution procedure. a: The inverted *E. coli* membrane containing F_oF_1 was labeled with FM. This F_oF_1 was ATPase-inactive because of the mutation (β-D242V) in F_1. b: The F_1 domain was stripped and removed by washing the membranes [32]. c: A His-tagged F_1 was added to reconstitute a functional F_oF_1 [32]. d: The F_oF_1 was solubilized by 0.05% lysolecithin and isolated with a Ni-NTA column [30]. The star indicates the fluorescence label.

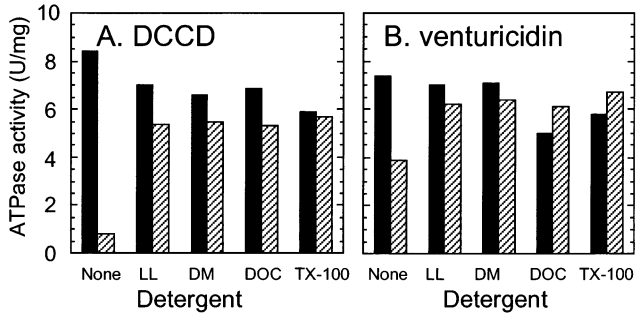

Fig. 4. Inhibition of ATPase activity of F_oF_1 by DCCD and venturicidin was apparently eliminated by detergents. A: F_oF_1 in 0.05% lysolecithin was incubated with (striped bar) or without (black bar) 40 μM DCCD, diluted 50-fold into the ATPase assay mixture containing the indicated detergent and the rate of ATP hydrolysis was measured. LL, 0.05% lysolecithin; DM, 0.1% dodecylmaltoside; DOC, 0.1% deoxycholate; TX-100, 0.24 mM Triton X-100. B: F_oF_1 in a very low concentration of lysolecithin (0.001%) was incubated in the ATPase assay mixture with (striped bar) or without (black bar) 70 μM venturicidin. After 2 min, the indicated detergent was added and ATPase activity was measured. The final concentrations of detergents were the same as those in A. Details of the experiments are described in Section 2.

the effect of lysolecithin on the DCCD sensitivity of F_oF_1 directly. F_oF_1 in 0.05% lysolecithin was incubated with 40 μM DCCD and diluted into the ATPase assay mixture. When the assay mixture did not contain lysolecithin, the detergent concentration in the F_oF_1 solution should be diluted to 0.001%. Under such conditions, the ATPase activity of DCCD-treated F_oF_1 was low, ~10% of that of the control F_oF_1, indicating more than 90% of the F_oF_1 activity was inactivated (Fig. 4A). However, when the assay mixture contained 0.05% lysolecithin, the same DCCD-treated F_oF_1 showed ~80% ATPase activity of that of the control F_oF_1; i.e. the apparent inhibition was only ~20%. Other detergents, deoxycholate (0.1%) and dodecylmaltoside (0.05%) had the same effect. The most disruptive in terms of uncoupling ATPase activity from the effect of DCCD in the F_o part was treatment with 0.24 mM Triton X-100. The enzyme had close to 100% of ATPase activity of the control F_oF_1 even after DCCD treatment. A similar effect of detergents was also observed for the inhibition by venturicidin, a non-covalent F_o specific inhibitor. In the trace amount of detergent (0.001% lysolecithin), 70 μM venturicidin inhibited ~50% of the ATPase activity of F_oF_1 (Fig. 4B). When F_oF_1 was in 0.05% lysolecithin or 0.05% dodecylmaltoside, the degree of the apparent inhibition was reduced to ~20%. In deoxycholate (0.1%) and Triton X-100 (0.24 mM), venturicidin did not inhibit, but rather activated the ATPase activity as has been reported previously [34,35].

4. Discussion

4.1. On the rotation of actin filament attached to F_oc

The results presented here establish that experiments designed to show rotation of the F_oc subunit ring in *E. coli* F_oF_1 give a positive result; rotation of the actin filament can be seen. Our findings are therefore in agreement with the recent observation of Sambongi et al. [22]. In our studies we observed rotation in around 1 in 1000 molecules in any field of view. Sambongi et al. observed the rotation in around 1 in 250 molecules. This is acceptable in single molecule studies given the treatments involved, if it can be convincingly demonstrated that the label (i.e. the actin filaments) are indeed attached to the subunit whose involvement is being examined *and* if the rotation can be shown to be a catalytic event. Reconstitution experiments were designed to examine rotation under conditions where the labeling of sites on F_1 can be ruled out. No rotations were now seen, suggesting that these might be at least in major part due to attachment of actin filament to F_1. Rotations contributed by the much more extensively labeled c subunits must be few in number and these as well as those due to γ are not inhibitor-sensitive. Such results point to disrupted coupling within F_oF_1, which we show is a detergent effect.

4.2. Disruptive effect of detergent on F_oF_1

For examining rotation in the presented single particle studies there is a need to disperse the F_oF_1. All of the detergents we tested for this dispersion caused disruption of the complex as measured by the loss of inhibitory effect of *both* DCCD and venturicidin, two inhibitors that block ATPase activity by binding to the F_o part, presumably blocking rotation. We found that the observed rotation was seen when enzyme was insensitive to DCCD but was not seen in fully coupled F_oF_1. There is previous data to show that DCCD is less effective in inhibiting the activity of F_oF_1 solubilized in detergent than membrane bound (lipid rich) F_oF_1.

More recently the disruptive effect of detergent on F_oF_1 has been exploited in X-ray crystallography studies of yeast F_oF_1 [20]. Crystals grown in a detergent, dodecylmaltoside, contained only F_1 and the F_oc subunit ring (10 copies), i.e. all of the other F_o subunits dissociated from the complex during crystallization. It is clear then that detergents can transform F_o into a 'non-native state' which causes functional uncoupling of F_oF_1 and often leads to further dissociation of F_o subunits. A non-native F_o state could result from impaired junction in the second stalk between F_ob and $F_1\delta$, a loosened connection between F_oa and F_oc and/or some loss of copies of F_oc. In most cases the effect of detergent is reversible, because detergent-solubilized F_oF_1 usually can recover the coupling function when incorporated into membranes, but it can be irreversible if F_o subunits are lost. Detergents solubilize hydrophobic proteins in membranes by loosening the lipid–protein contact but they can also weaken the contact between subunits in the complex. Even though our rotation assay was carried out in the absence of detergent, our preparation had been exposed to detergent during gel filtration.

4.3. Rotation of F_oc is not proven

Our findings are relevant to the recent study of Sambongi et al. [22], who used essentially the same material (*E. coli* F_oF_1) and strategy (actin filament presumably attached to F_oc), that we have employed. These workers also observed rotation that was detergent-dependent as they state that "the c subunit rotation could be observed in the presence of Triton X-100 but not in the absence of the detergent" [22]. As shown in Fig. 4, in the presence of Triton X-100, even at 0.24 mM in which they observed rotation, F_oF_1 is not intact in our hands and the coupling between F_o and F_1 is damaged. If the F_oc ring loses the connection to F_oa subunit as observed for yeast F_oF_1, the F_oc ring could rotate, by being dragged by the rotating γ. Obviously this rotation would be an artifact and unrelated to the events occurring in the intact F_oF_1. Sambon-

gi et al. described that the actin filament continued to rotate for 2 min then often disappeared [22]. This could be explained by a collapse process of the enzyme in detergent, i.e. the rotating γ forced the uncoupled F_oc ring to rotate with it until the loosened F_o sector fell apart and the actin filament then disappeared. In Triton X-100, F_oF_1 is not inhibited by DCCD (Fig. 4A). Instead of DCCD, Sambongi et al. presented the inhibitory effect of venturicidin on the rotation[1] as evidence that they observed the rotation of intact F_oF_1. However, they also report that their F_oF_1 preparation is relatively insensitive to venturicidin in as much as 'the inhibition of ATPase activity of F_oF_1 was ~10-fold lower than that reported previously'. The reported value was 65% [36] which means that inhibition was only 6.5%. We observe a slight activation by venturicidin in 0.24 mM Triton X-100 (Fig. 4B). There are previous studies to indicate that the ATPase activity of the Triton X-100 solubilized mitochondrial F_oF_1 is stimulated by venturicidin [34,35].

In summary there is little doubt that F_oF_1 works as a tiny rotary motor. The internal rotor includes the γ subunit and probably the ε subunit. It is attractive to postulate that γε drives the rotation of the F_oc subunit ring and vice versa. However, this remains to be proven conclusively.

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[1] Sambomgi et al. observed the pause-and-start type inhibition by venturicidin [22]. Overall velocity of rotation and comparison with bulk phase ATPase activity were not shown. Pauses were not very obvious by the eye in the figure in their paper. If pauses are really intrinsic nature of the rotation in the presence of venturicidin, analysis of frequency and duration of pauses will give k_{on} and k_{off} of venturicidin, respectively, and the pause position should distribute in the three-fold symmetry.