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F-ATPase: specific observation of the rotating **c** subunit oligomer of EF_oEF_1

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Abstract The rotary motion in response to ATP hydrolysis of the ring of c subunits of the membrane portion, F_o, of ATP synthase, F_oF_1, is still under contention. It was studied with EF_oEF_1 (*Escherichia coli*) using microvideography with a fluorescent actin filament. To overcome the limited specificity of actin attachment through a Cys-maleimide couple which might have hampered the interpretation of previous work, we engineered a 'strep-tag' sequence into the C-terminal end of subunit c. It served (a) to purify the holoenzyme and (b) to monospecifically attach a fluorescent actin filament to subunit c. EF_oEF_1 was immobilized on a Ni-NTA-coated glass slide by the engineered His-tag at the N-terminus of subunit β. In the presence of MgATP we observed up to five counterclockwise rotating actin filaments per picture frame of 2000 μm^2 size, in some cases yielding a proportion of 5% rotating over total filaments. The rotation was unequivocally attributable to the ring of subunit c. The new, doubly engineered construct serves as a firmer basis for ongoing studies on torque and angular elastic distortions between F_1 and F_o.

Key words: ATP synthase; Subunit **c**; EF_oEF_1; Single molecule; Rotation

1. Introduction

ATP synthases of bacteria, chloroplasts, and mitochondria utilize ion motive force for the synthesis of ATP from ADP and phosphate [1,2]. When operating in reverse (F-ATPase) the enzyme hydrolyzes ATP and generates ion motive force. The ATP synthase, in its simplest bacterial form, consists of eight different subunits, five in the F_1 portion, $(\alpha\beta)_3\gamma\delta\varepsilon$, and three in F_o, $\mathbf{ab_2c}_{10-12}$. The former catalyze substrate conversion and the latter are responsible for ion translocation. ATP hydrolysis by both isolated F_1 [3–9] and membrane-bound F_1 [10] drives the rotation of $\gamma(\varepsilon)$ relative to the $(\alpha\beta)_3$ barrel. The hypothesis that the counterpart of these rotor elements in F_1 is the **c** ring in F_o [2,11] has been based on X-ray crystal structure analysis [12–14] and cross-linking [15–20]. It has been suggested that the $\gamma\varepsilon$ assembly is rather rigidly connected with the ring of **c** subunits. Accordingly, subunits **a**–$\mathbf{b}_2$–δ–$(\alpha\beta)_3$ are considered to be the stator and subunits $\mathbf{c}_{10-12}$–γ–ε to be the rotor of the enzyme. Evidence for the corotation of subunits **c** and ε has been provided, although without time resolution, by chemical cross-linking [21]. Recently the group of M. Futai presented more direct evidence for the rotation of the **c** ring driven by ATP hydrolysis [22]. They used the video microscopy approach of Noji et al. [5]. The indicator, a fluorescent actin filament, was attached via streptavidin to an engineered and biotin-maleimide-modified cysteine on subunit **c**. As the maleimide function is not perfectly specific for cysteine, we aimed at a better approach to put ongoing studies on the nanomechanical properties of F_oF_1 on a firmer basis.

2. Materials and methods

2.1. Chemicals

Streptactin, streptactin-Sepharose, and desthiobiotin were purchased from IBA, Göttingen, Germany. Ni-nitrilotriacetic acid (NTA) horseradish peroxidase and Ni-NTA superflow were from Qiagen, Hilden, Germany. Biotin-$PEAC_5$-maleimide was from Dojindo, Japan. Phalloidin–tetramethylrhodamine B isothiocyanate conjugate was from Fluka, Buchs, Switzerland. All other reagents were of the highest grade commercially available.

2.2. Molecular genetics

A completely Cys-less clone carrying the His-tag sequence at the N-terminus of subunit β (RGSHHHHHHGM(-ATGKI...-β, pKH4) [8,23] was used as starting point. The Institut für Bioanalytik (Göttingen, Germany) carried out the cloning in order to add the *strep*-tag affinity peptide sequence (C-...MFAVA)-SWSHPQFEK C-terminal to subunit **c**. Successful cloning was checked by nucleotide sequencing. The resulting plasmid was called pSE1.

2.3. Preparation of EF_oEF_1

Escherichia coli strain DK8 [24] was transformed with pSE1. Cells were collected at $OD_{600} = 0.8$. Membranes were purified essentially according to Wise [25]. The procedure included two additional washing steps with 5 mM Tris–HCl (pH 8), 10 mM $MgCl_2$, 0.2 mM EGTA, 10% (v/v) glycerol followed by centrifugation at $220\,000 \times g$ for 90 min. Membrane pellet aliquots were stored (20 mg protein/ml in 5 mM Tris–HCl (pH 8), 10 mM $MgCl_2$, 70 mM KCl, 20% (v/v) glycerol) at −80°C. Membranes (100 mg protein) were suspended at 5 mg/ml protein in 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM K-ADP, 15% glycerol (buffer A), 1 tablet of Boehringer complete inhibitor tablets/50 ml extraction volume. *N*-Octyl-β-D-glucopyranoside (25% (w/v) in H_2O) was added dropwise to an initial concentration of 1%. After stirring for 15 min on ice additional detergent was added to a final concentration of 2.2% (w/v). Membrane protein was extracted by stirring on ice for 30 min followed by centrifugation at $100\,000 \times g$ for 90 min. Avidin was added (1 μg/ml) followed by dilution of the supernatant to 1% (w/v) *N*-octyl-β-D-glucopyranoside with buffer B (20 mM TES (pH 7.5), 5 mM $MgCl_2$, 1 mM K-ADP, 15% (v/v) glycerol). This solution was applied batchwise to 5 ml streptactin-Sepharose (settled volume, 5 mg streptactin/ml). The gel was packed into an empty NAP-10 column (Pharmacia), washed with 5 ml buffer B containing 1% (w/v) *N*-octyl-β-D-glucopyranoside at 0.5 ml/min. EF_oEF_1 was then eluted with 10 ml of the same buffer containing 2.5 mM desthiobiotin. EF_oEF_1 prepared from pKH4, i.e. lacking the *strep*-tag, did not bind to this column. Protein-containing fractions were combined and batchwise adsorbed onto 1 ml Ni-NTA superflow. The gel was packed into an empty NAP-5 column (Pharmacia), washed with 1 ml 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 1% (w/v) *N*-octyl-β-D-glucopyranoside, 20 mM imidazole, 10% (v/v)

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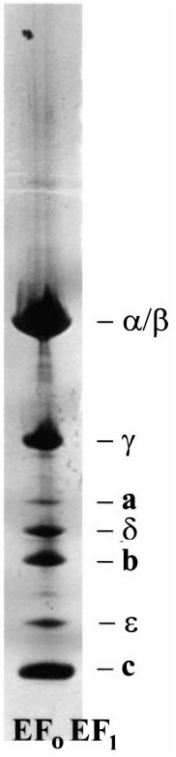

Fig. 1. SDS electrophoresis of doubly chromatographed EF_oEF_1 (200 ng) on 8–25% Pharmacia Phast Gel. Staining with Coomassie brilliant blue R250 followed by silver. This technique is, of course, by no means quantitative [27].

glycerol and eluted with the same buffer containing 150 mM imidazole resulting in 150 μg/ml protein. Protein determination was carried out according to Sedmak and Grossberg [26] and SDS electrophoresis with the Pharmacia Phast system (8–25% gradient gels). Staining was carried out with Coomassie followed by silver [27]. ATPase activity was measured with 0.1 μg protein, 50 mM Tris–HCl (pH 8.0), 3 mM $MgCl_2$, 10 mM Na-ATP, 1% *N*-octyl-β-D-glucopyranoside. Typical activities under these conditions were 70 U/mg.

2.4. Preparation of F-actin

G-actin was prepared from acetone powder obtained from rabbit skeletal muscle as described by Pardee and Spudich [28]. The protein was briefly sonified and gel filtrated against 2 mM MOPS/KOH (pH 7.0), 0.2 mM $CaCl_2$, 2 mM ATP (buffer G). Biotinylation was carried out with a six-fold molar excess of biotin-$PEAC_5$-maleimide for 2 h at ambient temperature. Excess reagent was removed by an overnight dialysis (buffer G) followed by two successive gel filtrations (Pharmacia PD 10/buffer G). This preparation was stored frozen in aliquots at −80°C. Prior to use, the thawed G-actin (18 μM) was converted into fluorescently labeled F-actin by addition of 20 mM MOPS/KOH (pH 7.0), 100 mM KCl, 10 mM $MgCl_2$, 18 μM phalloidin–tetramethylrhodamine B isothiocyanate conjugate (3 h/4°C) [5]. Special care was taken in pipetting the labeled F-actin. The filament length varied between 0.5 and 10 μm depending on the applied shear forces.

2.5. Immobilization of EF_oEF_1

Samples were filled into flow cells consisting of two coverslips (bottom, 26×76 mm^2; top, 21×26 mm^2) separated by parafilm strips. Protein solutions were infused in the following order (2×25 μl per step, 4 min incubation): (1) 0.8 μM Ni-NTA-horseradish peroxidase conjugate in 20 mM MOPS/KOH (pH 7.0), 50 mM KCl, 5 mM $MgCl_2$ (buffer R); (2) 10 mg/ml bovine serum albumin in buffer R; (3) 5 nM EF_oEF_1 in 50 mM Tris–HCl (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 10% (v/v) glycerol, 1% (w/v) *N*-octyl-β-D-glucopyranoside (buffer S); (4) wash with buffer S; (5) 0.5 μM streptactin in buffer S; (6) wash with buffer S; (7) 200 nM biotinylated, fluorescently labeled F-actin in buffer S (10 min incubation); (8) wash with buffer S; (9) 20 mM glucose, 0.2 mg/ml glucose oxidase, 50 μg/ml catalase, 0.5% 2-mercaptoethanol, 5 mM ATP in buffer S.

2.6. Video microscopy

An inverted fluorescence microscope (IX70, Olympus, Japan, lens PlanApo 100×/1.40 oil, fluorescence cube MWIG) was equipped with a silicon intensified tube camera (C 2400-08, Hamamatsu, Japan) and connected to a VHS-PAL video recorder (25 frames/s). With this setup filaments of 5 μm length appeared as 3 cm long rods on a 14″

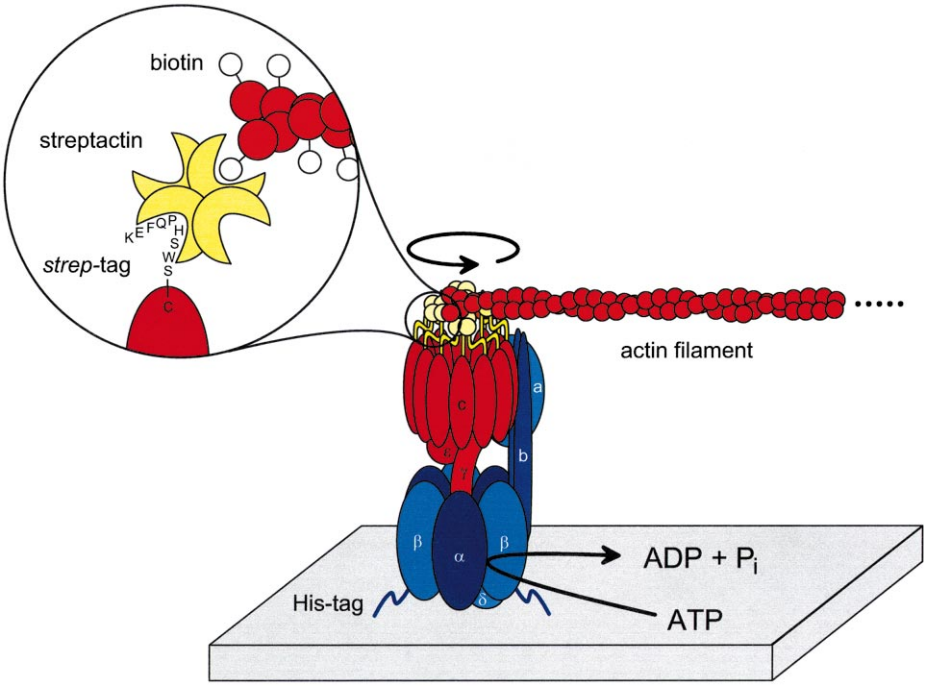

Fig. 2. Scheme of the experimental set-up. A fluorescently labeled actin filament–biotin–streptactin complex was connected to the engineered *strep*-tags of subunit **c** of the immobilized EF_oEF_1 molecule. Probably several *strep*-tags were needed to form a tightly binding spoke over the ring of subunit **c**. Subunits shown in blue are attributed to the stator, rotating components are drawn in red and yellow.

monitor. The flow cell was loaded with freshly extracted and chromatographed samples of EF_oEF_1, labeled with actin filaments and single molecule rotation was studies up to 30 min after loading. Video data were captured (frame grabber FlashBus, Integral Technologies, USA) and further processed by using the software ImagePro4.0 (Media Cybernetics, USA).

3. Results and discussion

Fig. 1 shows a silver-stained SDS–PAGE of the EF_oEF_1 preparation after consecutive *strep*-tag (first) and His-tag affinity chromatography (second). All eight subunits were

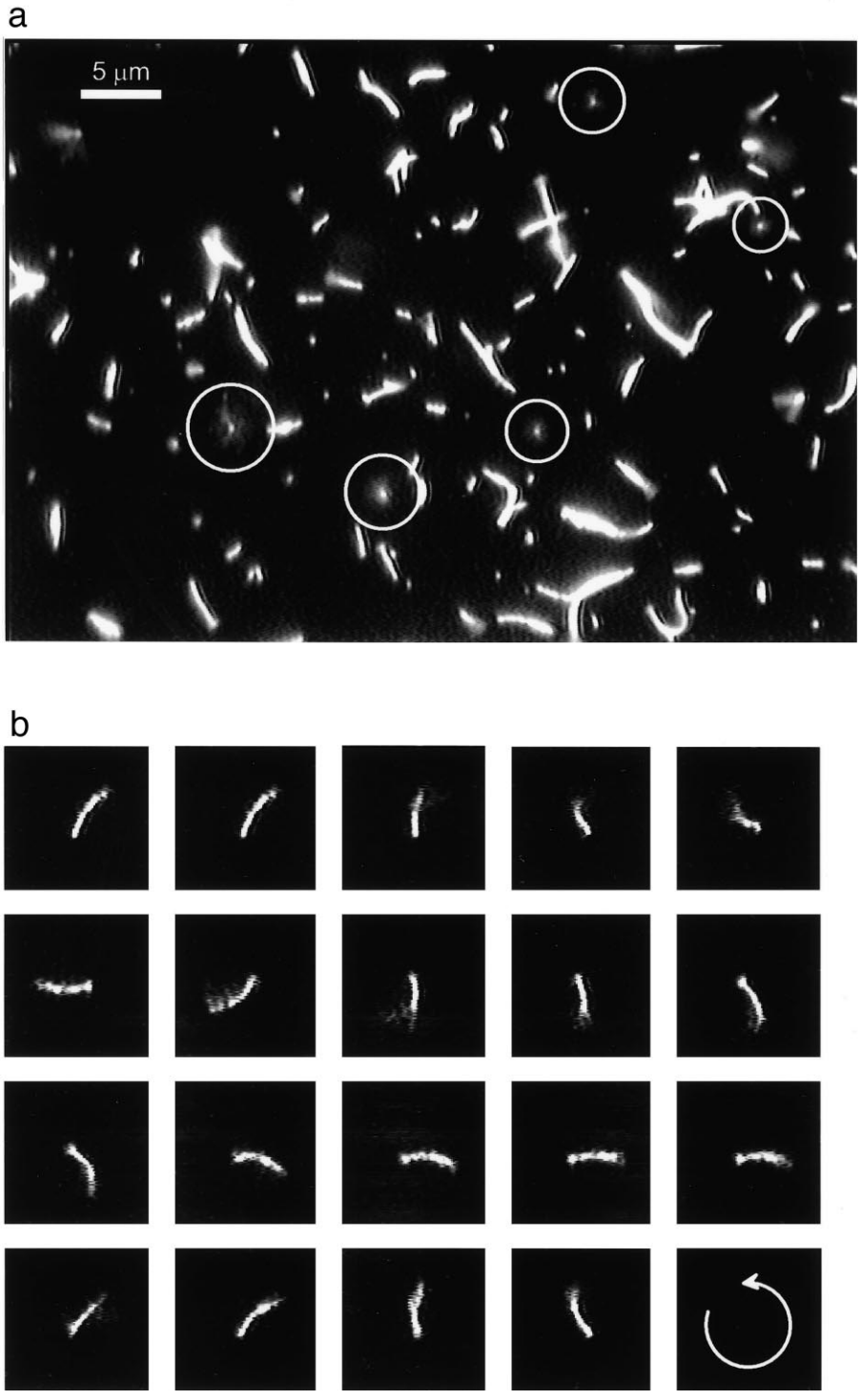

Fig. 3. a: Superimposition of 25 subsequent frames (52×36 μm^2, 40 ms duration each). The five indicated rotating actin filaments appear as blurred circular spots. b: Sequential images of a rotating actin filament attached to the **c** subunit oligomer on EF_oEF_1. The filament length was 2.7 μm and the averaged rotational speed was 0.44 rounds/s in the presence of 5 mM MgATP; every fourth frame was selected so that the time interval between images was 160 ms. This figure can be viewed as a time-lapse movie sequence at our web site (http://131.173.26.96/se/se.html).

present. Inversion of the given order of chromatographic steps (first His- then *strep*-tag) produced the loss of subunits α and β. Samples prepared by only using *strep*-tag affinity chromatography also yielded positive results in the rotation assay, but the occurrence of rotating single molecules was lower.

Fig. 2 illustrates the experimental set-up. Basically we followed the original protocol by Noji et al. [5] as also used in [22]. However, by genetically engineering the *strep*-tag affinity peptide sequence into the C-terminus of subunit **c** we avoided the chemical modification of EF_oEF_1 with biotin-maleimide. This was necessary since we had observed (not documented) non-specific labeling in several subunits (including γ!) upon treatment with tetramethylrhodamine-6–maleimide of the Cys-less mutant of EF_oEF_1 (obtained from plasmid pKH4 [8]). If such a side reaction occurs on subunits γ or ε the actin filament is connected to the 'wrong' target, namely EF_1 instead of EF_oEF_1. The observed rotation might then be erroneously attributed to the **c** ring. By using two genetically engineered tags we circumvented such problems and additionally gained purification quality (His-tag and *strep*-tag affinity chromatography) with the consequence of improved yield of rotating molecules. This is documented in Fig. 3.

Fig. 3a shows a viewing field of 52×36 μm containing a total of about 100 actin filaments. The picture represents the superimposition of 25 video frames of 40 ms duration each. Five filaments, i.e. an astoundingly high 5% of total, were rotating (encircled). They appear blurred in the superimposition of Fig. 3a. Fig. 3b shows a series of movie frames for one particular filament. The direction of the rotation (counterclockwise) was evident from the curvature of the filament, which was elastically deformed under the influence of torque generated by the enzyme and counteracted by the viscosity of the medium. It is noteworthy that in several cases the rotation of the filament continued up to the fading of the fluorescence, i.e. for several minutes. The high probability of detecting rotating filaments by the *strep*-tag assay aiming at subunit **c** practically excluded artifacts caused by solitary EF_1.

In order to further discriminate against EF_1-dependent artifacts we checked this method in different ways. (1) Deliberate omission of either one single component of the chain Ni-NTA-horseradish peroxidase–*strep*-tagged EF_oEF_1–streptactin–biotin-F-actin prevented the binding of fluorescent F-actin, as evident from the absence of fluorescent filaments within the flow cell. (2) EF_1 preparations (both Cys-containing and Cys-free), which were not treated with biotin-maleimide, did not immobilize fluorescent actin filaments at all. (3) Importantly, *streptactin*, a genetically engineered streptavidin, *could not substitute for streptavidin in the rotation assay* (carried out as in [5]) *with biotinylated EF_1 and fluorescent actin*. In a side by side experiment with the very same preparation of biotinylated EF_1^{pKH7} (γ_{K109C} [8]) and binding of the filaments via streptactin or streptavidin, respectively, we observed many *rotating* filaments with streptavidin but none with streptactin, although many filaments were *immobilized* in both cases. One reason for streptactin to only allow for binding of resting but not of rotating filaments may be the three point mutations in streptactin as compared with streptavidin, which cause different electrophoretic mobilities of the two proteins and suggest slightly different tertiary structures [29]. Another reason might be the binding of more than one molecule of streptactin to the **c** ring thus allowing for several contacts with biotin on the actin filament and thereby strengthening the interactions between the filament, streptactin, and the engineered EF_oEF_1. It is also conceivable that the much weaker binding affinity between streptactin and biotin allowed for attachment of the actin filament, but as soon as rotation of the filament started, it was torn off. The binding affinity between biotin and streptavidin is in the range of 10^{-14} M, whereas that of biotin/

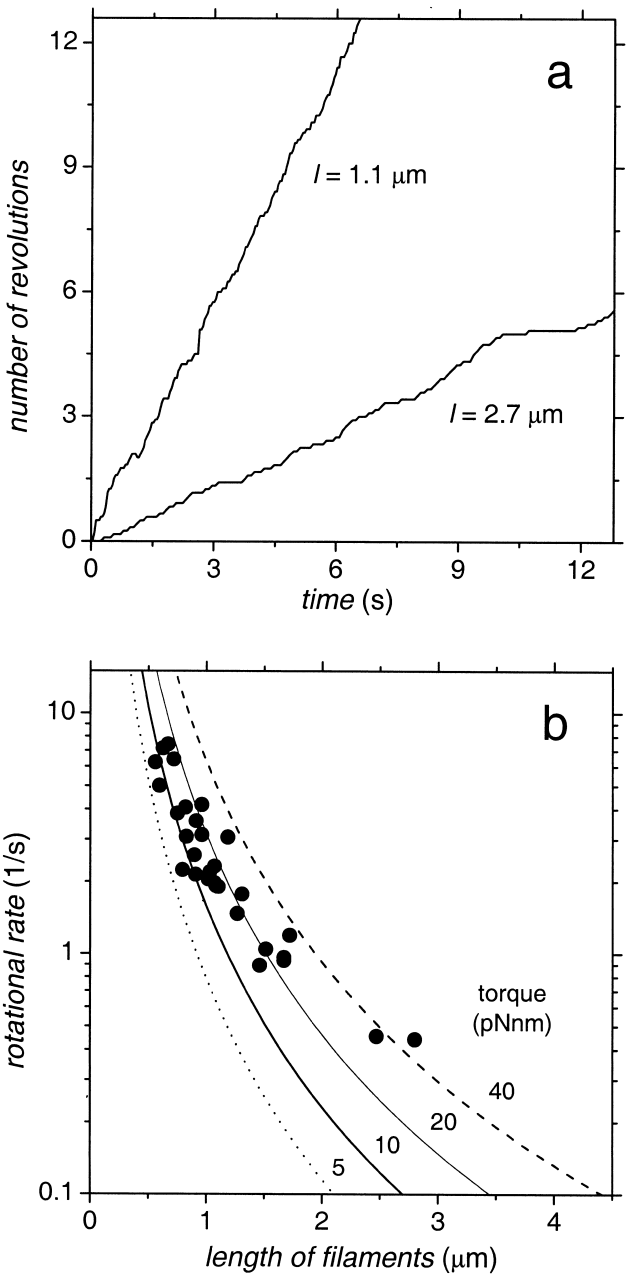

Fig. 4. a: Two typical time courses of the rotation of subunit **c** oligomer. Images of rotating actin filaments in the presence of 5 mM MgATP were recorded with a silicon intensified tube camera (C 2400-08, Hamamatsu) and analyzed with the ImagePro software. b: Rotational speed in revolutions per second as a function of the length of the actin filaments. Frictional torque was calculated with $T=(4\pi/3)\omega\eta L^3/(\ln(L/2r)-0.447)$, where ω is angular velocity; η is 10^{-3} Nsm^{-2}, the viscosity of the medium; L is the length of the actin filament; and r is 5 nm, the radius of the actin filament [32,33]. Lines show the rotational speed ($v=\omega/2\pi$) under a constant torque of 5 pN nm (dotted line), 10 pN nm (thick line), 20 pN nm (thin line), and 40 pN nm (dashed line).

streptactin is estimated to be 1000-fold lower (Dr. Thomas Schmidt, IBA Göttingen, personal communication). (4) Analogously, streptavidin could not substitute for streptactin in the $EF_oEF_1^{pSE1}$ rotation assay, exactly as expected (cf. (3) above). Taken together the high yield of rotating filaments (up to 5%) and the above-mentioned controls excluded that we inadvertently observed the rotation of subunits γ and/or ε in solitary EF_1 instead of that of subunit **c** of EF_oEF_1.

Fig. 4a shows two typical time courses of rotation of the **c** ring in EF_oEF_1 and Fig. 4b shows the rotational rate as a function of filament length. The decline of the rate as a function of filament length reproduced the behavior previously reported for the rotation of subunit γ in solitary EF_1 [5–8]. The calculated torque as a function of filament length (see curves in Fig. 4b) is in the same range as previously reported. A fine analysis of the torque as function of the angular displacement is in progress.

Our approach has established beyond doubt that the rotation of the **c** ring of F_o was observed, as driven by ATP hydrolysis in F_1. It cannot, however, prove that every rotating **c** ring resulted from a complete EF_oEF_1, i.e. one with one copy of subunit **a** and two copies of subunit **b**, which form the stator holding EF_o and EF_1 together (see Fig. 2). The recently resolved crystal structure of yeast mitochondrial (F_oF_1), where the equivalents of *E. coli* subunits **a** and $\mathbf{b}_2$ were lacking, has demonstrated the stability of a $(\alpha\beta)_3\gamma\varepsilon\mathbf{c}$ ring structure without **a** and $\mathbf{b}_2$ [13]. It is well possible therefore that we observed the rotation of the **c** -ring in such 'incomplete' EF_oEF_1. Still, the corotation of γε with the **c** ring is now firmly established and it is very difficult to imagine how the observed ATP hydrolysis-dependent rotations of subunits γ, ε, and **c** are related to the fully coupled function of F_oF_1 if these three subunit (assemblies) together do *not* constitute the rotor.

The new, doubly engineered construct may serve as a firmer basis for ongoing studies on torque and functional elastic distortions between F_1 and F_o [30,31].

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