ATP synthases (F₀F₁-ATPases) mechanically couple ion flow through the membrane-intrinsic portion, F₀, to ATP synthesis within the peripheral portion, F₁. The coupling most probably occurs through the rotation of a central rotor (subunits αβγαβ) relative to the stator (subunits αβγ). The translocation of protons is conceived to involve the rotation of the ring of ε subunits (the ε oligomer) containing the essential acidic residue εD61 against subunits αβ. In line with this notion, the mutants εD61N and εD61G have been previously reported to lack proton translocation. However, it has been surprising that the membrane-bound mutated holoenzyme hydrolyzed ATP but without translocating protons. Using detergent-solubilized and immobilized EF₀F₁ and by application of the microvideographic assay for rotation, we found that the ε oligomer, which carried a fluorescent actin filament, rotates in the presence of ATP in the mutant εD61N just as in the wild type enzyme. This observation excluded slippage among subunit γ, the central rotary shaft, and the ε oligomer and suggested free rotation without proton pumping between the oligomer and subunit α in the membrane-bound enzyme.

ATP synthases of bacteria, chloroplasts, and mitochondria use ion-motive force for the synthesis of ATP from ADP and phosphate (1–4). When operating in reverse (F₁-ATPase), the enzyme hydrolyzes ATP and generates ion-motive force. ATP synthases (F₀F₁-ATPases) mechanically couple ion flow through the membrane-intrinsic portion, F₀, to ATP synthesis within the peripheral portion, F₁. The coupling most probably occurs through the rotation of a central rotor (subunits αβγαβ) relative to the stator (subunits αβγαβ). The translocation of protons is conceived to involve the rotation of the ring of ε subunits (the ε oligomer) containing the essential acidic residue εD61 against subunits αβ. In line with this notion, the mutants εD61N and εD61G have been previously reported to lack proton translocation. However, it has been surprising that the membrane-bound mutated holoenzyme hydrolyzed ATP but without translocating protons. Using detergent-solubilized and immobilized EF₀F₁ and by application of the microvideographic assay for rotation, we found that the ε oligomer, which carried a fluorescent actin filament, rotates in the presence of ATP in the mutant εD61N just as in the wild type enzyme. This observation excluded slippage among subunit γ, the central rotary shaft, and the ε oligomer and suggested free rotation without proton pumping between the oligomer and subunit α in the membrane-bound enzyme.

Material and Methods

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Germany). Oligonucleotide primers were synthesized by MWG Biotech (Ebersberg, Germany). Streptactin-Sepharose was purchased from IBA (Gottingen, Germany). Nickel-nitrilotriacetic acid (Ni-NTA) horseradish peroxidase and Ni-NTA SuperFlow were from Qiagen (Hilden, Germany). Biotin-PEAC5-maleimide was from Dojindo (via Gerbu Bio-technologies, Göttingen, Germany). The Lumi-Light Western blotting reagent was obtained from Roche Molecular Biochemicals. Venturicidin A was obtained from Dr. B. Liebermann (Department of Pharmacology, Univer-
sity of Jena, Jena, Germany), but the supply exhausted in the meantime. Other reagents were of the highest grade commercially
available.

Molecular Genetics—The complete cysteine-less plasmid pSE1 (β-His
tag, Strep-tag at the C terminus of c) (15) was used as starting material. Site-directed mutagenesis was performed by standard PCR using the oligonucleotide primers 5'-ATTCTGATTTGGTGTGGTGACG3'-3', 5'-ATGGGATGAGCATCCAGACCAATCG-3', 5'-GGTACGCGCC-
AGTACCTTAAATCTTCAG-3', and 5'-GGTTGCTGTAATTGCTATGC-
CGATGCCG-3'. The BamHI/XhoI fragment of pSE1 was substituted with the corresponding fragment carrying the cD61N muta-
tion by restriction and religation. Successful cloning was confirmed by nucleotide sequencing. The resulting plasmid was called pKG1. pKG1 carried a His6 tag at the N terminus of subunits β, a C-terminal Strep-tag at
subunit c, a point mutation in subunit c (D61N), and all of the Cys residues were replaced by Ala (24).

Preparation of EF0EF1—E. coli strain DK5 (25) was transformed with pKG1, and cells were grown on minimal medium containing 10%
(v/v) LB and 0.5% (w/v) glucose. Cells were collected at A600 with pKG1, and cells were grown on minimal medium containing 10%
available. The complete cysteine-less plasmid pSE1 (β-His
tag, Strep-
tag at its C terminus. The desired point mutation within subunit
was obtained from Roche Molecular Biochemicals. Venturicidin A was obtained from Dr. B. Liebermann (Department of Pharmacology, University of Jena, Jena, Germany), but the supply exhausted in the meantime. Other reagents were of the highest grade commercially available.

Molecular Genetics—The complete cysteine-less plasmid pSE1 (β-His tag, Strep-tag at the C terminus of c) (15) was used as starting material. Site-directed mutagenesis was performed by standard PCR using the oligonucleotide primers 5'-ATTCTGATTTGGTGTGGTGACG3'-3', 5'-ATGGGATGAGCATCCAGACCAATCG-3', 5'-GGTACGCGCCAGTACCTTAAATCTTCAG-3', and 5'-GGTTGCTGTAATTGCTATGC- CGATGCCG-3'. The BamHI/XhoI fragment of pSE1 was substituted with the corresponding fragment carrying the cD61N mutation by restriction and religation. Successful cloning was confirmed by nucleotide sequencing. The resulting plasmid was called pKG1. pKG1 carried a His6 tag at the N terminus of subunits β, a C-terminal Strep-tag at subunit c, a point mutation in subunit c (D61N), and all of the Cys residues were replaced by Ala (24).

Preparation of EF0EF1—E. coli strain DK5 (25) was transformed with pKG1, and cells were grown on minimal medium containing 10% (v/v) LB and 0.5% (w/v) glucose. Cells were collected at A600 = 0.8. The membranes were isolated and purified essentially according to Wise (26), and membrane proteins were extracted as described previously (15). After the addition of avidin, the octylglycoside extract of membranes from 25 g of collected cells containing 140 mg of total membrane protein was diluted with buffer A (20 mM TES (pH 7.5), 5 mM MgCl2, 1 mM K-ADP, 15% (v/v) glycerol) to 1% octylglycoside (total volume, 100
ml) and then was applied batchwise to 5 ml of streptactin-Sepharose (settled volume, 5 ml streptactin/ml). Washing and elution were performed as described previously (15). Protein-containing fractions (2 mg of protein) were combined, and batchwise was adsorbed onto 1 ml of Ni-NTA Superflow. After washing, up to 100 μg of pure EF0EF1 eluted from the column. Protein determinations were carried out according to
Sedmak and Grossberg (27) and SDS-gel electrophoresis with the Pharm-
cacia Phast system (8–25% gradient gels). Staining was carried out with Coomasie Blue followed by silver (28). ATPase activity was measured with 0.1 μg of protein, 50 mM Tris/HCl (pH 8.0), 3 mM MgCl2, 10 mM Na-ATP, 1% N-octyl-β-D-glucopyranoside. Rotating Assay—Samples were filled into flow cells consisting of two coverslips (bottom, 26 × 76 mm2; top, 21 × 26 mm2, thickness, 0.15 mm (Menzel-Gläser/ProLabor, Georgsmarienhu-
te, Germany) separated by parafilm strips. Protein solutions were infused in the following order (2 × 25 μl 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 MgCl2 (buffer B); 2) 10 mg/ml bovine serum albumin in buffer B; 3) 5–10
mg/ml Venturicidin A (buffer C); 4) 50 mM Tris/HCl (pH 7.5); 50 mM MgCl2, 10 mg of bovine serum albumin/ml, 10% (v/v) glycerol, 1% (w/v) N-octyl-β-D-glucopyranoside (buffer C); 4) wash with buffer C; 5) 0.5 μg streptactin in buffer C; 6) wash with buffer C; 7) 200 mM biotinylated fluorescent-labeled F-actin (15 min incubation); 8) wash with buffer C; and 9) 20 mM glucose, 0.2 mg/ml glucose oxidase, 50 μM/ml catalase, 5 mM ATP in buffer C. The deliberate omission of either one single component of the chain Ni-NTA-horseradish peroxidase, EF0EF1-, streptactin, and biotin-F-actin prevented the binding of fluorescent F-actin as evident from the absence of fluorescent filaments within in the flow cell. This ensured that the actin filaments were attached to subunit(s) c in the correct manner. Also, the rotating filaments only could be observed in the presence of ATP (15), whereas in its absence (or with ADP present), this number dropped to zero without affecting the number of immobilized filaments.

Video Microscopy—An inverted fluorescence microscope (IX70, lens PlanApo 100x/1.40 oil, fluorescence cube MWIG, Olympus, Hamburg, Germany) was equipped with a silicon-intensified tube camera (C2400-08, Hamamatsu, Herrsching, Germany) and connected to a VHS-PAL video recorder (25 frames/s). With this setup, the filaments of 5-μm length were recorded as 3-cm long rods on a 14-inch monitor. A freshly
chromatographed sample of EF0EF1 was loaded into the flow cell and labeled with fluorescent actin filaments. The rotation of single filaments was observed for up to 3 min. A single molecule rotation was followed up to 30 min after loading. Video data were captured (frame grabber FlashBus, Integral Technologies, Indianapolis, IN) and further processed using the software ImagePro Plus 4.0 (Media Cybernetics, Silver Spring, MD) and Matlab 5.2 (The Math Works, Natick, MA).

Other Methods—ATPase activity was measured at protein concentrations of 10 μg/ml in 50 mM Tris/HCl (pH 8.0), 3 mM MgCl2, 10 mM Na-ATP, 1% octylglycoside. After incubation for 5 min at 37 °C, the reaction was stopped by the addition of trichloroacetic acid, and the released Pi was determined colorimetrically (29).

RESULTS AND DISCUSSION

EF0EF1 mutant SE1 (15) was used as starting material. In this mutant, all wild type cysteines are substituted by alanines (24), each β subunit carries an engineered His6 tag at its N terminus, and each c subunit carries an engineered Strep-tag at its C terminus. The desired point mutation within subunit c (Asp61→Asn) was introduced by PCR and confirmed by nucleotide sequencing. The resulting plasmid was called pKG1. Because the cd61N mutation causes uncoupling, EF0EF1-KG1 had to be prepared from cells grown on medium supplemented with LB and glucose. This yielded 30–100 μg of EF0EF1-KG1/8 l culture volume. Typical activities after purification were 90 units/mg. Fig. 1 shows the results of an SDS-electrophoresis with purified EF0EF1-KG1, EF0EF1-SE1, and a control (EF0EF1-KH7) (11) after purification by streptactin-Sepharose and nickel-nitrilotriacetic acid affinity chromatography. Phamaica Phast gradient gel 8–25% silver/silicon tungstic acid stain (28) was used. The size difference between subunits c from EF0EF1-KG1, EF0EF1-SE1, and EF0EF1-KH7 is because of the C-terminal Strep-tag engineered into EF0EF1-KG1 and EF0EF1-SE1.
tained with EF₀EF₁-KG1. Panel B shows the dependence of the filament rotational rate from filament length. It is evident that EF₀EF₁-S1 (15) and EF₀EF₁-KG1 were indistinguishable.

How do these results complement the proposal that detergent solubilized FₐFᵥ is uncoupled from proton control (16), possibly by partial displacement of subunits a and b from their locations in the native enzyme (20)?

The exact structural consequences of the cD61N mutation are not known. They are expected to be small, because both the size and the polarity of Asp and Asn are very similar. Still the lack of an essential protonable group is sufficient to completely block proton conductance in both directions, passive under ATP synthesis and actively driven by ATP hydrolysis (23).

Assuming that ATP synthesis is driven by the rotation of subunits γεcₐ, the failure to conduct protons is expected to prevent both rotation and ATP synthesis. However, ATP hydrolysis catalyzed by the membrane-bound enzyme is only diminished but not completely blocked (by 50% in the cD61N mutant and not at all in the cD61G mutant (23)). This finding in view of the structure of FₐFᵥ either implies some sort of displacement of subunits γε from their c oligomer counterpart (with FₐFᵥ still kept together by the stator subunits a, b, and d) or continued corotation of γεcₐ without concomitant proton pumping. The latter is the case as we show here. Thus, “uncoupling” in EF₀EF₁-KG1 is brought about by ATP hydrolysis-driven freewheeling of the c oligomer.

The interaction of subunits γε and the c oligomer both in the wild-type enzyme and the mutant EF₀EF₁-KG1 withstands the strong mechanical strain between the ATP-hydrolyzing motor and either the drag force exerted on the actin filament or in situ the proton-motive force. In the cD61N mutant, the interactions between γε and the c oligomer are expected to be as strong as in the wild type enzyme, because the mutation is comparatively small and not likely to affect FₐFᵥ interactions at a distance of around 2.7 nm. Accordingly, we did not observe a more pronounced tendency of Fₐ to dissociate from Fᵥ than with the wild type enzyme during preparation (data not shown).

To summarize, 1) the membrane-bound cD61N mutant hydrolyzes ATP without proton translocation; 2) the γε-c oligomer interactions are strong enough to withstand considerable mechanical strain; and 3) solubilized wild type and mutant enzyme rotate γεcₐ upon ATP hydrolysis. These findings together indicate ATP hydrolysis-driven rotation of the c oligomer not only with solubilized but also with membrane-bound enzyme and irrespectively of the native or non-native location of subunits a and b. The expected steric hindrances for the rotation of the c oligomer relative to subunits a and b would be smallest for the cD61G mutant and perhaps a little more pronounced for the cD61N mutant in accordance with the reported ATPase activities of the respective membrane-bound mutant enzymes (23).

How do these implications relate to the assumed rotary mechanism of FₐFᵥ? Proton transport through the Fₐ portion of ATP synthase relies at least on two essential amino acid residues, Asp-61 on subunit c and Arg-210 on subunit a (E. coli numbering). A mechanism on how proton translocation might drive the rotation of the ring of c subunits (the c oligomer) relative to subunits a and b has been detailed previously (1, 3, 4, 17–20).

This model now would seem to be valid for all ATP synthases, because the proposed location of the acidic residue in subunit c of the sodium translocating ATP synthase close to the cytoplasmic side of the membrane (31) had to be abandoned as shown by recent cryoelectron microscopic data.

The model features four assumptions. 1) The acidic residue cD61 is positioned at the center of the membrane. It is accessible for protons from both aqueous phases by two parallel but laterally off-set access channels. 2) There is a stochastic rotation of the c oligomer relative to subunit a driven by thermal impact (Langevin force). 3) It is limited by an electrostatic constraint, namely that the acidic residue on subunit c (Asp-61) is forcedly electroneutral (protonated) when facing the lipid core. 4) It is forcedly anionic (deprotonated) when opposing the permantly positively charged residue aR210, which is juxtaposed cD61 (for a detailed discussion cf. Refs. 31–33). The c oligomer thus rotationally fluctuates relatively to subunit a and progresses in one single direction by protonation of one Asp – through one channel followed by the loss of another proton from a protonated Asp into the other channel located at the opposite side of the membrane. The model implicitly assumes that the interacting essential side chains are properly oriented without the requirement of large protein flexibility other than the thermal motion of the “rigid” c oligomer relative to subunit a.

This model both explains wild type features and the behavior of the cD61N mutant, i.e. the loss of passive and active proton translocation along with conservation of the ATPase activity of the membrane-bound enzyme, which correlates the c oligomer with or without proton pumping. However, the occurrence of the corotation in the mutant in vivo contradicts the fourth proposal above, because the postulated transient but essential
these subunits count the proposed rotation of the helix with Asp-61 in subunit c. The observations become better understandable by taking into account with expectations as predicted from the model and experiments, ar210A does not pump protons but allows for passive proton translocation (34). However, its membrane-bound ATP hydrolysis activity is largely inhibited. Because the mutation does not affect the F1 part, the only explanation for this inhibition would be the blockage of the c oligomer rotation. These observations become better understandable by taking into account the proposed rotation of the helix with Asp-61 in subunit c and with Arg-210 in subunit R relative to the other helices in these subunits “swiveling” (35). Proton translocation by F0 would seem to involve both intersubunit as well as intrasubunit rotational movements.

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