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F_1-ATPase, the C-terminal End of Subunit γ Is Not Required for ATP Hydrolysis-driven Rotation*

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ATP hydrolysis by the isolated F_1-ATPase drives the rotation of the central shaft, subunit γ, which is located within a hexagon formed by subunits $(\alpha\beta)_3$. The C-terminal end of γ forms an α-helix which properly fits into the "hydrophobic bearing" provided by loops of subunits α and β. This "bearing" is expected to be essential for the rotary function. We checked the importance of this contact region by successive C-terminal deletions of 3, 6, 9, 12, 15, and 18 amino acid residues (*Escherichia coli* F_1-ATPase). The ATP hydrolysis activity of a load-free *ensemble* of F_1 with 12 residues deleted decreased to 24% of the control. EF_1 with deletions of 15 or 18 residues was inactive, probably because it failed to assemble. The average torque generated by a *single molecule* of EF_1 when loaded by a fluorescent actin filament was, however, unaffected by deletions of up to 12 residues, as was their rotational behavior (all samples rotated during 60 ± 19% of the observation time). Activation energy analysis with the ensemble revealed a moderate decrease from 54 kJ/mol for EF_1 (full-length γ) to 34 kJ/mol for $EF_1(\gamma\text{-}12)$. These observations imply that the intactness of the C terminus of subunit γ provides structural stability and/or routing during assembly of the enzyme, but that it is not required for the rotary action under load, proper.

ATP is the universal free energy currency of prokaryotic and eukaryotic cells. It is synthesized in mitochondria, chloroplasts, and the cytoplasm of prokaryotic cells by F_0F_1-ATP synthase (*cf.* Refs. 1–6 for recent reviews). The enzyme works like a (reversible) rotary molecular machine with two motors/generators mounted on a common shaft and hold together by an eccentric stator (7–11). In ATP synthesis mode the F_0 part translocates protons, thereby converting protonmotive force into the mechanical energy of rotary motion. Rotation is forwarded through the shaft into the F_1 part where it drives ATP synthesis. In ATP hydrolysis mode the rotation is reversed, and ions are pumped through F_0 in the opposite direction. The *Escherichia coli* enzyme (EF_1),[1] has the simplest subunit composition. It consists of eight different subunits, five in the peripheral F_1 portion and three in the membrane-intrinsic F_0, with stoichiometries of $(\alpha\beta)_3\gamma\delta\epsilon$ for F_1 and probably $\mathbf{ab}_2\mathbf{c}_{10}$ for F_0 (12). In view of the rotary mechanism they also can be organized into "rotor" ($\gamma\epsilon\mathbf{c}$) and "stator" ($\alpha\beta\delta\mathbf{ab}$). According to the crystal structure of bovine heart mitochondrial F_1 (13) the C-terminal region of subunit γ properly fits into a supposed "hydrophobic bearing" formed by loops in the upper portion of the hexagon of subunits $(\alpha\beta)_3$. Multiple sequence alignments showed that this region of γ is more conserved than the remainder (14, 15). One would expect therefore that truncations, point mutations, and covalent cross-links between the "bearing" and the rotor should inhibit the activity. But this expectation was not always met. 1) EF_1 with truncated γ (lacking 10 C-terminal residues) was still active (15). 2) The ATPase activity of the homologous enzyme from chloroplasts (CF_1) tolerated truncations of γ up to 20 C-terminal deletions, 10–16 residue truncations even resulted in activation of the ATP hydrolysis activity (16). 3) Point mutations in the C-terminal region of *E. coli* γ were tolerated in many cases, including some that changed polar residues into hydrophobic ones or even caused a charge reversal (15). 4) A number of second site mutations were identified within the region of residues 269–280 in *E. coli* γ, which restored energy coupling (17) in the significantly impaired mutants γM23R or γM23K. These constructs, however, were not able to build up protonmotive force to the extent of wild type enzyme despite comparable levels of ATPase activity (18) and despite generation of the same apparent torque (19). These restoring second site point mutations often resulted from the substitution of bulky residues with smaller ones, but in one case Ala was substituted with Val, thus increasing the occupied volume of the side chain significantly (17). Later, segments were identified in γ by suppressor mutagenesis and second site mutagenesis, which are separated in the three-dimensional structure but still restored energy coupling if combined (20). 5) The effects of a deleterious frameshift in *E. coli* γ could be mended by point mutations in subunit β, at quite a distance from the frameshift region within γ (Thr^{277}–Val^{286} (21)). 6) Most surprisingly, a covalent link between the penultimate C-terminal residue of EF_1-γ and a nearby residue of α (γA285C ↔ αP280C (22)) neither inhibited ATP hydrolysis nor the rotation of subunit γ relative to $(\alpha\beta)_3$ and the torque generation under load. It would appear that the C-terminal part of γ does play an important role in ATP synthase, but according to the foregoing not to the extent of certain residues being absolutely required. The situation is reminiscent of the "DELSEED" sequence in subunit β, which, despite conservation among many species, still tolerated not only one single point mutation (7) but even complete substitution of the acidic residues by alanines (23). The pronounced interplay of the rotor subunit γ with its partners α and β is underlined by the fact that revertants map to distant regions not only located on the defective γ itself, but also on β.

In the above cited work with truncated subunit γ the activity of the enzyme constructs has been measured by ATP hydrolysis

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[1] The abbreviations used are: EF_1, *E. coli* F_1-ATPase; $EF_1(\gamma\text{-}x)$, EF_1 with subunit γ lacking x C-terminal amino acid residues (x = 3, 6, 9, 12, 15, 18); CF_1, chloroplast F_1-ATPase; Ni-NTA, nickel-nitrilotriacetic acid; MOPS, 4-morpholinepropanesulfonic acid.

FIG. 1. **Schematic representation of *E. coli* F_1.** Two copies each of subunits α and β are omitted for clarity. γ is depicted in *pale gray* with the truncations of γ shown in *gray* and *black*. Subunit α is on the *left*, and subunit β is on the *right* in *pale gray*.

assay with isolated and solubilized F_1. Since a mechanical load is absent under these conditions, the F_1-ATPase operates under kinetic control. In the holoenzyme, F_0F_1, on the other hand, F_1 works against the ion-driven F_0 motor. Likewise, in the micro-videographic rotation assay (10) F_1 works against the (viscous) drag of an actin filament of micrometer length. Under both conditions the enzyme is mechanically strained, and turnover is greatly slowed down or even stalled (thermodynamic control). It has been an interesting question whether F_1 constructs with truncated γ are still able to operate in the rotation assay, where mechanical strain might cause the rotor to get jammed in the bearing. This prompted us to examine the functional importance of the C-terminal end of *E. coli* subunit γ. We deleted 3, 6, 9, 12, 15, and 18 amino acid residues from its C terminus (Fig. 1) and determined the rotation and the torque generated by these truncated mutants. We also assayed the ATPase activity, however, with emphasis on the Arrhenius activation energy. The results show that up to 12 C-terminal amino acid residues of subunit γ are not required for the rotary function of the enzyme. During rotation phases of the loaded enzyme the torque remained the same as in controls. In the freely running enzyme, however, the activation energy of the truncated constructs decreased from 54 to 34 kJ/mol, as if the activation barrier was determined by a mechanical constraint that was lessened by truncation. Although there was little effect on the functioning of assembled enzyme molecules, their structural stability was affected by the truncation of γ, and the removal of 15 or 18 amino acids probably prevented assembly altogether.

EXPERIMENTAL PROCEDURES

Chemicals and Enzymes—All enzymes were obtained either from New England Biolabs (Frankfurt/Main, Germany), MBI Fermentas (St. Leon-Rot, Germany) or Sigma (Taufkirchen, Germany). Oligonucleotide primers were custom-synthesized by MWG-Biotech (Ebersberg, Germany). Biotin-$PEAC_5$-maleimide (Dojindo, Japan) was obtained via the German representative Gerbu Biotechnik, Gaiberg, Germany. Nickel-nitrilotriacetic acid (Ni-NTA) superflow and nickel-nitrilotriacetic acid horseradish peroxidase were obtained from Qiagen (Hilden, Germany). All other reagents used were of the highest grade available commercially.

Molecular Genetics—Starting with plasmid pKH7 (all wild type cysteines substituted by Ala (24), His_6-tag extension at the N terminus of subunit β, γK109C (25)) plasmid pMM4 was generated by transferring the *Kpn*I/*Sac*I fragment of pKH7 (containing the entire coding region of uncG and part of the sequence of uncD (Ala^1–Leu^{162}) into pBluescript II SK +/−. Based upon a method described by Weiner *et al.* (26), deletions were introduced in uncG by PCR with the following 5′-phosphorylated oligonucleotides as 3′-5′-primers: 5′-P-GGCCCCCGAGACGATCTCG-GTGAGTTCCTG-3′, 5′-P-GACGATCTCGGTGAGTTCCTGAGTAAT-GCTGG-3′, 5′-P-GGTGAGTTCCTGAGTAATGCTGGCCTGACG-3′, 5′-P-CTGAGTAATGCTGGCCTGACGAGCTTTGTTG-3′, 5′-P-GCTG-GCCTGACGAGCTTTGTTGTATACC-3′, 5′-P-ACGAGCTTTGTTG-TATACCAACTGCAGCTC-3′, and as 5′-3′-primer the 5′-phosphorylated oligonucleotide 5′-P-TAAACAGGTTATTTCGTAGAGGAT TTAATATGAG-3′ (Fig. 2). pMM4 was used as PCR template in all cases. *Kpn*I/*Sac*I fragments of pMM4 subclones containing the *uncG* deletions were thereafter reintroduced into pKH7 yielding the following plasmids: pMM16 ($\gamma\Delta Ala^{284}$–Val^{286}, "γ-3"), pMM20 ($\gamma\Delta Ser^{281}$–Val^{286}, "γ-6"), pMM17 ($\gamma\Delta Glu^{278}$–Val^{286}, "γ-9"), pMM8 ($\gamma\Delta Glu^{275}$–Val^{286}, "γ-12"), pMM18 ($\gamma\Delta Ile^{272}$–Val^{286}, "γ-15"), and pMM19 ($\gamma\Delta Gln^{269}$–Val^{286}, "γ-18"). All mutations were confirmed by sequencing (27). The resultant plasmids were transformed into *E. coli* strain DK8 (28), which contains an *uncB-uncC* deletion and hence has lost the ability to functionally express F_0F_1-ATPase. The mutants were tested for expression of a functional ATP synthase by growth on solid succinate medium (34 mM KH_2PO_4, 64 mM K_2HPO_4, 0.3 mM $MgSO_4$, 20 mM $(NH_4)_2SO_4$, 1 μM $FeSO_4$, 1 μM $ZnCl_2$, 10 μM $CaCl_2$, 50 μg/ml each of L-isoleucine, L-asparagine, L-valine, and thymine, 2 μg/ml thiamine, 0.4% (w/v) sodium succinate, and 3% (w/v) agar).

Expression and Purification of EF_1—Transformed strains were grown in glycerol-containing minimal medium (34 mM KH_2PO_4, 64 mM K_2HPO_4, 0.3 mM $MgSO_4$, 20 mM $(NH_4)_2SO_4$, 1 μM $FeSO_4$, 1 μM $ZnCl_2$, 10 μM $CaCl_2$, 50 μg/ml each of L-isoleucine, L-asparagine, L-valine, and thymine, 2 μg/ml thiamine, and 0.5% (v/v) glycerol). Before inoculation ampicillin was added to a final concentration of 100 μg/ml. Cells were harvested at A_{600} = 0.8. Membrane preparation and EF_1 isolation were carried out essentially as described previously (29). Cells were not treated with phenylmethylsulfonyl chloride and pancreatic DNase before the Ribi press passage. Instead the buffer for resuspension of the cells after harvesting was supplemented with an EDTA-free protease inhibitor mixture (Roche Diagnostics, Mannheim, Germany, 1 tablet/100 ml). The crude supernatant of the final wash step (containing the F_1-ATPase) was applied to an anion exchange column (Tosoh Fractogel TSK-DEAE 650(S), Toyo Soda, Darmstadt, Germany) equilibrated with buffer A (50 mM Tris/H_2SO_4, 10% (v/v) methanole, pH 7.8). Using a stepwise salt elution by addition of buffer B (buffer A + 0.5 M Na_2SO_4), EF_1 was eluted in the 75–150 mM Na_2SO_4 fraction (10–20 mg of protein/12 liters of culture volume). After addition of 1 mM MgATP the protein was precipitated with 3.2 M $(NH_4)_2SO_4$ and stored at 4 °C.

Rotation Assay—2–3 mg protein were purified from the $(NH_4)_2SO_4$ precipitate by gel filtration through NAP-10 columns (Amersham Biosciences, Freiburg, Germany), which were equilibrated with 20 mM MOPS-KOH, 5 mM $MgCl_2$, 50 mM KCl, pH 7.0 (buffer C). After determination of the protein concentration the 20-fold molar excess of Biotin-$PEAC_5$-maleimide was added, and the samples were incubated 15–20 min at room temperature. The protein was then subjected to Ni-NTA affinity chromatography. After equilibration with buffer C the biotinylated EF_1 was eluted with buffer C containing 150 mM imidazole yielding typically 250 μg of protein/500 μl. For the rotation experiments flow cells were constructed of two coverslips (bottom 26 × 76 mm, top 21 × 26 mm, thickness 0.15 mm (Menzel-Gläser/ProLabor, Georgsmarienhütte, Germany), which were 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 C); 2) 10 mg/ml bovine serum albumine in buffer C, (buffer D); 3) 5–10 nM EF_1 in buffer D; 4) wash with buffer D; 5) 0.5 μM streptavidin in buffer D; 6) wash with buffer D; 7) 200 nM biotinylated, fluorescently labeled F-actin (30) in buffer D (7 min incubation); 8) wash with buffer D; 9) 20 mM glucose, 0.2 mg/ml glucose oxidase, 50 μg/ml catalase, 5 mM ATP in buffer D (aiming at the torque generated by the mutant enzyme with truncated γ this high concentration was chosen to bypass occasional lack of substrate). Rotating filaments were observed with an inverted fluorescence

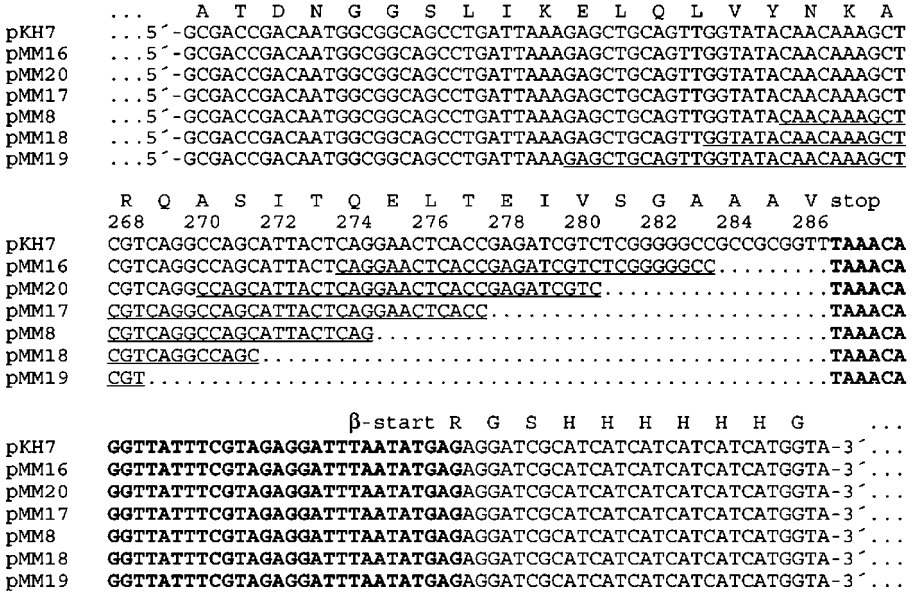

FIG. 2. **Nucleotide and amino acid sequences of the C-terminal parts of γ and the N-terminal parts of β of the control and $EF_1(\gamma\text{-}x)$.** The 5′-3′-primer sequences used for the truncations are shown in *boldface*, and complementary sequences are *underlined*.

microscope (IX70, Olympus, Japan; lens PlanApo 100×/1.40 oil, fluorescence cube MWIG) and recorded with a VHS-PAL video recorder at 25 frames/s. The search for rotating filaments was carried out for 30 min per flow cell. Video data were captured (frame grabber FlashBus, Integral Technologies), and filament length as well as rotation velocity were analyzed with ImageProPlus 4.0 (Media Cybernetics). Deliberate omission of either one single component of the required components (Ni-NTA-horseradish peroxidase-biotinylated EF1-streptavidin-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 specifically to subunit γ.

Arrhenius Analysis—Samples for Arrhenius analyses were prepared just as the samples for the rotation assay, except that they were not biotinylated. After Ni-NTA affinity chromatography 1 mM MgATP was added to the eluate. ATP hydrolysis activity was measured spectrophotometrically in the presence of an ATP-regenerating system essentially according to Ref. 31. The reaction mixture contained 25 mM Tris/HCl, 25 mM KCl, 2 mM $MgCl_2$, 5 mM KCN, 2 mM phoshoenolpyruvate, 5 mM ATP, 0.35 mM β-NADH, 30 units/ml L-lactate-dehydrogenase, and 30 units/ml pyruvate kinase, pH 7.5. 1 ml of the reaction mixture was preincubated 10 min at the desired temperature, then the reaction was started by addition of 1 μg of EF_1. The decrease of NADH absorption at 340 nm was recorded for 5–30 min.

Other Procedures—Polyacryamide gel electrophoresis in the presence of SDS was carried out in the Amersham Biosciences Phast system (Amersham Biosciences). Protein bands were stained with Coomassie Brilliant Blue R-250 (32) and quantified using ImageProPlus 4.0 (Media Cybernetics). Protein determinations were performed according to Sedmak and Grossberg (33).

RESULTS AND DISCUSSION

Construction of uncG Deletion Mutants and Purification of $EF_1(\gamma\text{-}x)$—Aiming at the function of the C terminus of ATP synthase subunit γ, we constructed six mutants of *E. coli* F_0F_1-ATPase containing C-terminally truncated γ subunits (γ-3, -6, -9, -12, -15, and -18) by substitution of the *Kpn*I/*Sac*I-fragment of pKH7 (carrying the entire coding sequence for subunit γ (25)) with the corresponding fragments of mutated subclones (*cf.* Fig. 2). The resulting plasmids, pMM16 (γ-3), pMM20 (γ-6), pMM17 (γ-9), pMM8 (γ-12), pMM18 (γ-15) and pMM19 (γ-18), were transformed into *E. coli* DK8 (28) and tested for growth on succinate (Table I). It would seem that in all cases, except for $EF_1(\gamma\text{-}15)$ and $EF_1(\gamma\text{-}18)$, the complexes were assembled but that they became increasingly unstable. A slight increase of the doubling times (EF_1-KH7 (control) → $EF_1(\gamma\text{-}12)$) paralleled a decrease of the F_1-ATPase activities of solubilized $EF_1(\gamma\text{-}x)$. Prolonged doubling times of $EF_1(\gamma\text{-}15)$ and $EF_1(\gamma\text{-}18)$ as well as the failure of the respective mutants to grow on succinate suggested that these strains were not able at all to assemble functional EF_0EF_1. These cells had to rely on glycolysis for the synthesis of ATP, just as *E. coli* strain DK8 without any plasmid. In comparison with $EF_1(\gamma\text{-}15)$ and $EF_1(\gamma\text{-}18)$ the significantly smaller doubling time of *E. coli* DK8 might be explained by the complete lack of plasmids and therefore a less demanding nucleotide metabolism.

The assumption that $EF_1(\gamma\text{-}15)$ and $EF_1(\gamma\text{-}18)$ did not assemble was supported by the failure to isolate measurable amounts of F-ATPase. On the other hand, the yields of truncation mutants up to "γ-12" and the control were comparable. The purity of the resulting EF_1 preparations was checked by SDS-polyacryamide gel electrophoresis (Fig. 3). It was evident that all EF_1 preparations had the same α:β:γ ratio. The ATP hydrolysis activities of the purified $EF_1(\gamma\text{-}x)$ at 35 °C decreased with increasing deletion length (Table I). $EF_1(\gamma\text{-}3)$ showed 70%, $EF_1(\gamma\text{-}6)$ 50%, $EF_1(\gamma\text{-}9)$ 30%, and $EF_1(\gamma\text{-}12)$ 24% of the hydrolysis activity of the control (EF_1-KH7). These results were consistent with those previously reported by Iwamoto *et al.* (15) who found 63 and 14% of the *membrane* ATPase activity of a control at 37 °C for $EF_1(\gamma\text{-}4)$ and $EF_1(\gamma\text{-}10)$, respectively. $EF_1(\gamma\text{-}18)$ in their hands also was not capable of growing by oxidative phosphorylation (15).

Effect of Deletions at the C Terminus of γ on the Torque of EF_1 (Loaded by F-actin)—The effects of C-terminal truncations of γ on the torque (Fig. 4) were investigated by measuring the rotational velocities of an attached actin filament (10) dependent on its length. It is noteworthy that the probability to find *rotating* filaments decreased proportionally with the truncation length: Within 30 min around 36 rotating filaments were observed with EF_1-KH7, 15 with $EF_1(\gamma\text{-}3)$, 6 with $EF_1(\gamma\text{-}6)$, 2 with $EF_1(\gamma\text{-}9)$, and just 1 with $EF_1(\gamma\text{-}12)$.

The *apparent* torque generated by single enzyme molecules was calculated according to Refs. 10 and 34 using the following equation (see Ref. 35),

$$T = \left(\frac{4\pi}{3}\right)\left(\frac{2\pi\nu\eta L^3}{\ln\left(\frac{L}{r}\right) - 0.447}\right) \qquad \text{(Eq. 1)}$$

where T denotes the torque, ν the rotational rate, η the viscosity of the ambient medium, L the filament length, and r the radius of the actin filament (2.8 nm). The assumption in the cited work that the rotating filament operated against the

TABLE I

Growth, doubling times, yields, ATPase activities, and activation energies of EF_1-KH_7 and $EF_1(\gamma$-x)

Strains were spread on medium with succinate as sole carbon source and incubated three days at 37 °C. For determination of doubling times 100 ml of glycerol-containing minimal medium were inoculated with 10 ml of an overday LB culture of the corresponding mutant. After 16-h incubation at 37 °C these cultures were used to inoculate 1 liter of glycerol-containing minimal medium to A_{600} = 0.1. Ampicillin was added to a final concentration of 100 μg/ml. Since *E. coli* DK8 (without any plasmid) lacks ampicillin resistance, tetracycline was used to a final concentration of 10 μg/ml. A_{600} was controlled up to 12 h. The activation energies for EF_1-KH_7 and $EF_1(\gamma$-x) were calculated from the slopes of the plots shown in Fig. 6 for the range between 20 and 35 °C.

E. coli DK8 complemented with the indicated plasmid	Growth at 37 °C on solid succinate medium	Doubling times at 37 °C in glycerol-containing minimal medium	EF_1 yield per liter glycerol-containing minimal medium	Hydrolysis activity of F_1 at 35 °C, pH 8	Activation energy (E_a) at 30 °C
		min	*mg/liter*	*units/mg*	*kJ/mol*
		139			
pKH7 (control)	+	139 ± 6	1.5 ± 0.3	93 ± 8	54 ± 8
pMM16 (γ-3)	+	145 ± 11	1.5	65 ± 6	41 ± 6
pMM20 (γ-6)	+	153 ± 8	1.2	46 ± 4	36 ± 5
pMM17 (γ-9)	+	156 ± 4	1.2	28 ± 2	35 ± 5
pMM8 (γ-12)	+	157 ± 10	0.8 ± 0.2	22 ± 2	34 ± 5
pMM18 (γ-15)		202			
pMM19 (γ-18)		230			

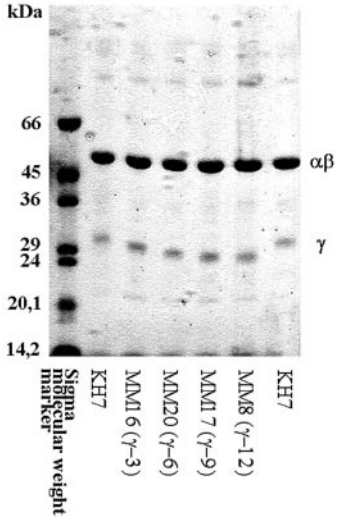

FIG. 3. **SDS-polyacrylamide gel electrophoresis (12.5% homogeneous gel, Amersham Biosciences Phast system) in the presence of 2% (w/v) sodium dodecyl sulfate stained with Coomassie Brilliant Blue R-250.** Protein concentration was 1 mg/ml; each line contains 0.3 μg of protein.

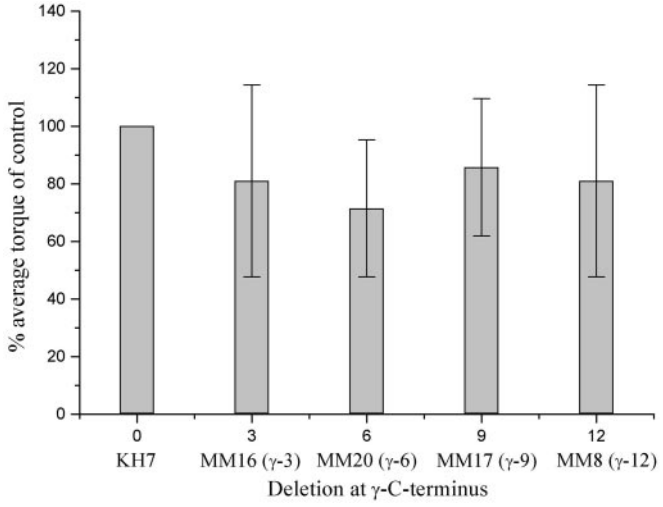

FIG. 4. **Comparison of the maximal average torques generated by EF_1-KH7 and $EF_1(\gamma$-*x*) during rotation.** The average torque of the control (51 pN nm) was set to 100%. The decrease of the average torques in $EF_1(\gamma$-*x*) was not significant.

viscous drag of the bulk medium as determined by the viscosity of bulk water, namely $\eta(H_2O) = 10^{-3}$ kg m^{-1} s^{-1}, is questionable. We have previously scrutinized this notion by gauging the torque by the filament's curvature, as with a spring balance (36, 37). The result, a value of about 50 pN nm, has been 2–3-fold larger than inferred from measurements of the rotation velocity in the same assay (see Table I in Ref. 37). One reason for this higher torque is that the viscosity at the surface is higher than in the bulk due to the immediate vicinity of filament and surface (38). It is important to note that the high torque implies a higher than previously thought value of the free energy per 120° angular progression (*i.e.* per one molecule of ATP hydrolyzed), namely 50 pN nm·2π/3, and this matches the free energy of ATP hydrolysis under the given experimental conditions, *i.e.* 105 pN nm or 63 kJ/mol.

In the present work we used the handier semiquantitative torque analysis by rotation rate and corrected the obtained value by one and the same factor by reference to the free energy of ATP hydrolysis under the given experimental conditions (ΔG = 63 kJ/mol).

Fig. 5 shows the rotational velocity of filaments as function of their length in various $EF_1(\gamma$-*x*) and in the control. The line shows the rate expected for a torque of 51 pN nm. Within

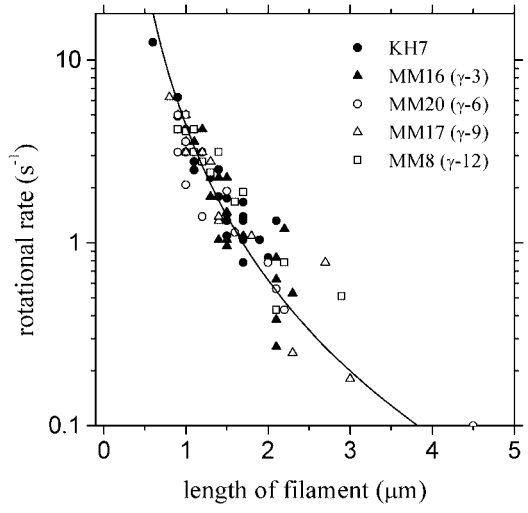

FIG. 5. **Rotational rate dependent on filament length for EF_1-KH7 and $EF_1(\gamma$-*x*).** The "isotorque" at 51 pN nm was calculated with an apparent surface viscosity $\eta = 2.4 \cdot 10^{-3}$ kg/(ms). The plot contains data of "whips" (filament end attached to the enzyme) as well as of "propellers" (middle attachment). For propellers the torque was calculated for each propeller blade and added to yield the total torque. The data resulted from the following numbers of rotating filaments: 23 (control), 25 (γ-3), 15 (γ-6), 11 (γ-9), and 14 (γ-12).

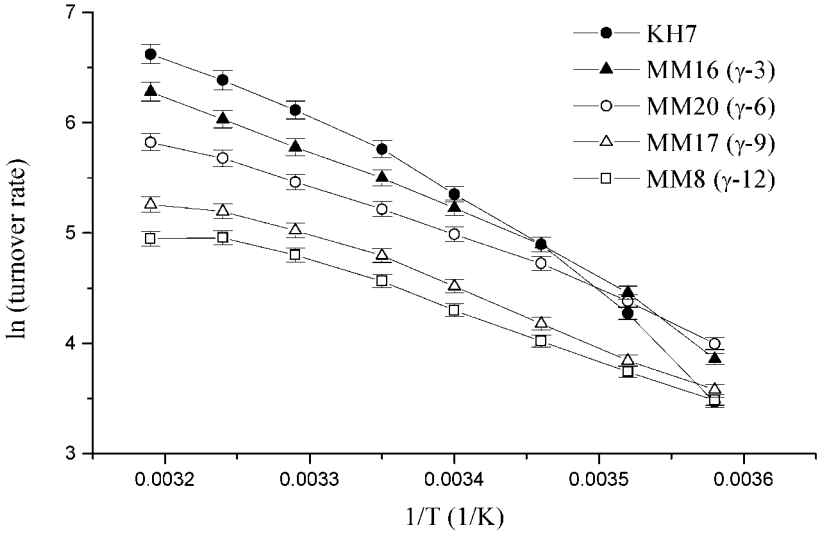

FIG. 6. **Arrhenius plot of the log rate of ATP hydrolysis over the reciprocal temperature for truncated EF_1 and the control.** Measurements were performed with an ATP-regenerating system. 1 μg of protein was added to 1 ml of reaction mixture, and the decrease of NADH absorption was followed at a wavelength of 340 nm for up to 30 min. Specific enzyme activities were calculated from the linear areas by using a molar absorption coefficient $\epsilon_{NADH} = 6.22 \cdot 10^6$ cm^2/mol (at 340 nm and room temperature). One unit is equivalent to 1 μmol $ATP_{hydrolyzed}$/min. Turnover numbers were calculated using a molecular mass of 384,000 daltons for EF_1. Activation energies were calculated from the slopes for the range around 30 °C and are shown in Table I.

scattering limits the average torque was similar for all samples including the control. Observation times were limited to 3 min by bleaching of the tetramethylrhodamine-phalloidine-labeled actin filaments. The perpetuation of rotation did not significantly differ between $EF_1(\gamma\text{-}x)$ and the control. Observation "windows" (as defined by finding a rotating filament and continuing the observation until the rotation either stopped completely or the filament was torn off) typically lasted from 42 to 115 s. Rotation occurred for 60 ± 19% of these times. Both the frequency and the duration of stops were indistinguishable between the control (EF_1-KH7) and $EF_1(\gamma\text{-}x)$. The decreased ATPase activity of the truncation mutants obviously was not caused by more frequent lapses into the Mg-ADP-inhibited state (39).

The average torque was neither dependent on the length of γ nor on the ATP hydrolysis activity. Since the attached actin filament slowed down rotation by orders of magnitude (as compared with the load-free enzyme), the enzyme operated close to thermodynamic equilibrium. In this view it even was not expected that effects on the kinetic parameters, say on V_{max}, would bear on the rotation rate under load and thereby on the torque.

Effect of Deletions at the C Terminus of γ on the Activation Energy of EF_1 (Load-free Enzyme)—The turnover of the load-free enzyme was expected to reveal the effects of truncation of subunit γ on the kinetic properties of the enzyme. Fig. 6 shows Arrhenius plots of the rate of ATP hydrolysis in the presence of an ATP-regenerating system. The temperature was varied between 5 and 40 °C. Whereas the Arrhenius plots for $EF_1(\gamma\text{-}12)$ and $EF_1(\gamma\text{-}9)$ were almost linear over the entire temperature range (with a minor decrease at temperatures above 35 °C), the hydrolysis activities of the control (EF_1-KH7) and of $EF_1(\gamma\text{-}3)$ decreased at low (5–15 °C) and high temperatures (above 40 °C (data not shown)). At close to physiological temperature (~30 °C) the activity was the lower the greater the truncation was. It was impossible to decide whether this was caused by a smaller fraction of active enzyme molecules in the ensemble of truncated enzymes or to a decrease of the pre-exponential factor. Since the activation energies were not very different in the same temperature range, we assumed that the number of active molecules decreased with increasing truncation length.

We compared the respective activation energies in the temperature range around 30 °C. The truncated enzyme, $EF_1(\gamma\text{-}x)$, showed lower activation energy (*cf.* Table I) than the control, *e.g.* 34 kJ/mol ($EF_1(\gamma\text{-}12)$) as compared with 54 kJ/mol (EF_1-KH7). This was consistent with, but did not prove, a weakened interaction of the C terminus of γ with $(\alpha\beta)_3$. In other words, the partial removal of the supposed bearing did not result in increased internal friction.

It is noteworthy that the decrease of active molecules in proportion to the length of truncation was not related to the Mg-ADP-inhibited form of F-ATPase (*cf.* above (39)). Active $EF_1(\gamma\text{-}x)$ seemed to operate just like the control, regardless of the truncation length (up to 12). But the probability to reversibly switch into an inactive state increased dependent on the truncation length. The interplay between the rotor and stator parts of ATP synthase would seem to comprise the entire structure, not just the interacting surfaces of rotor and stator. The chloroplast enzyme differs from the *E. coli* enzyme by different N-terminal portions of subunits α and β conferring, *e.g.* tentoxin sensitivity to the spinach enzyme (40). These portions of chloroplast α and β may increase the interactions between $(\alpha\beta)_3$ and γ in CF_1 to an extent even allowing for a 20-residue truncation to be tolerated. In contrast in EF_1 15 residues lacking at the C terminus of γ prevent assembly.

Lowered activation energies implied that the activation barrier was more closely related to the mechanical contacts between γ and $(\alpha\beta)_3$ than to the events in the three catalytic sites and at α-β subunit interfaces. It was conceivable that the major obstacle for free (activation-less) angular motion of γ was the contact with the hydrophobic bearing, which was removed (lowered) upon truncation of γ. A remarkable and reproducible feature of the Arrhenius plot (Fig. 6) was that the activities of EF_1-KH7 and $EF_1(\gamma\text{-}3)$ crossed over those of $EF_1(\gamma\text{-}6,9,12)$ at low temperature. This effect was not due to greater cold instability of the control, since cold inactivation was reversible. Increased friction then is a likely explanation. Under these conditions product dissociation would not be rate-limiting (41, 42) but perhaps the nucleotide binding affinity change brought about by extensive conformational changes (4). Without a crystal structure of the truncated enzyme this remains speculation, though. The evolutionary constraint responsible for the sequence conservation at the C-terminal end of γ might be just structural stability and/or routing of assembly rather than mechanical function, as mentioned. A similar development might be reflected in the fact that the three noncatalytic nucleotide binding sites apparently are dispensable for ATP hydrolysis yet have been maintained during evolution (43–45).

The "lubricated" rotation of the C-terminal end of subunit γ in the hydrophobic bearing at the top of $(\alpha\beta)_3$ has been an attractive concept (13). An alternative view has emerged from our previous finding that a covalent connection between the

C-terminal end of subunit γ and the top of α neither inhibits the hydrolytic activity of EF_1 nor the rotation of γ (22). Based thereupon it has been speculated that a swivel joint may be located in that portion of γ. One surprising result of the present study is that both the holding and the bearing function are dispensable, since the entire region of γ can be deleted without affecting rotation.

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