F\textsubscript{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\textsubscript{1}-ATPase drives the rotation of the central shaft, subunit γ, which is located within a hexagon formed by subunits α and β. 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 (\textit{Escherichia coli} F\textsubscript{1}-ATPase). The ATP hydrolysis activity of a load-free ensemble of F\textsubscript{1} with 12 residues deleted decreased to 24% of the control. EF\textsubscript{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\textsubscript{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\textsubscript{1} (full-length γ) to 34 kJ/mol for EF\textsubscript{1}(γ\textsubscript{3−12}). 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\textsubscript{0}F\textsubscript{1}-ATP synthase (\textit{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\textsubscript{0} part translocates protons, thereby converting protonmotive force into the mechanical energy of rotary motion. Rotation is forwarded through the shaft into the F\textsubscript{1} part where it drives ATP synthesis. In ATP hydrolysis mode the rotation is reversed, and ions are pumped through F\textsubscript{0} in the opposite direction. The \textit{Escherichia coli} enzyme (EF\textsubscript{1}),\textsuperscript{*} has the simplest subunit composition. It consists of eight different subunits, five in the peripheral F\textsubscript{1} portion and three in the membrane-intrinsic F\textsubscript{0}, with stoichiometries of (αβ\textsubscript{3}γ\textsubscript{5}) for F\textsubscript{1} and (γ\textsubscript{3}) for F\textsubscript{0} (12). In view of the rotary mechanism they also can be organized into “rotor” (γγε) and “stator” (αβ\textsubscript{3}αβ\textsubscript{3}ε). According to the crystal structure of bovine heart mitochondrial F\textsubscript{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 (αβ\textsubscript{3}γ). 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\textsubscript{1} with truncated γ (lacking 10 C-terminal residues) was still active (15). 2) The ATPase activity of the homologous enzyme from chloroplasts (CF\textsubscript{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 \textit{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 \textit{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 \textit{E. coli} γ could be mended by point mutations in subunit β, at quite a distance from the frameshift region within γ (Thr\textsuperscript{277}→Val\textsuperscript{296} (21)). 6) Most surprisingly, a covalent link between the penultimate C-terminal residue of EF\textsubscript{1}-γ and a nearby residue of α (γA285C ↔ αP280C (22)) neither inhibited ATP hydrolysis nor the rotation of subunit γ relative to (αβ\textsubscript{3}γ) 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 “DELSSEED” 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 alamines (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 by the payment of page charges. This article must therefore be hereby underlined by the fact that revertants map to distant regions not only located on the defective γ itself, but also on β.

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* The abbreviations used are: EF\textsubscript{1}, \textit{E. coli} F\textsubscript{1}-ATPase; EF\textsubscript{1}(γγε), EF\textsubscript{1} with subunit γ lacking C-terminal amino acid residues (x = 3, 6, 9, 12, 15, 18); CF\textsubscript{1}, chloroplast F\textsubscript{0}F\textsubscript{1}-ATPase; Ni-NTA, nickel-nitrilotriacetic acid; MOPS, 4-morpholinepropanesulfonic acid.
assay with isolated and solubilized F₁. Since a mechanical load is absent under these conditions, the F₁-ATPase operates under kinetic control. In the holoenzyme, F₅F₃F₂F₁, on the other hand, F₁ works against the ion-driven Fₒ motor. Likewise, in the micro-videographic rotation assay (10) F₁ works against the (viscous) drag of an actin filament of micrometer length. Under both conditions the enzyme is mechanically strained, and turn-over is greatly slowed down or even stalled (thermodynamic control). It has been an interesting question whether F₁ 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), MBF Fermentas (St. Leon-Rot, Germany) or Sigma (Taufkirchen, Germany). Oligonucleotide primers were custom-synthesized by MWG-Biotech (Ebersberg, Germany). Biotin-PEAC₅-maleimide (Dajindo, Japan) was obtained via the German representative Gerbu Biotechnik, Gaiberg, Germany. Nickel-nitriolactric acid (Ni-NTA) superflow and nickel-nitriolactric 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 cyto-}

tines substituted by Ala (24), His₆-tag extension at the N terminus of subunit β, y109C (25) plasmid pMM4 was generated by transfransferring the KpnI/SacI fragment of pKH7 (containing the entire coding region of uncG and part of the sequence of uncD (Aala²-Leu²)² 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’-GGCCCGAGAGCACCTCGTGGTACCTGT-3’, 5’-GACGATTCGTTGAGTCTGGATGTAAGTCGTGG-3’, 5’-GGTTGAGTTCTGTAATGCTGGCCTGACG-3’, 5’-CTGATGAAATGCTGGCAGAGCTTGTG-3’, 5’-GCTGGCAGCGAGTTGTTGTAATACCTGACGTC-3’, and as 5’-3’-primer the 5’-phosphorylated oligonucleotide 5’-AAACAGGTTTACCTGAGGATTTAATAGGAG-3’ (Fig. 2). PMM4 was used as PCR template in all cases. KpnI/SacI fragments of pMM4 subclones containing the uncG deletions were thereafter reintroduced into pKH7 yielding the following plasmids: pMM16 (γAla²⁵⁰⁻²⁵⁰Val²⁵⁰, γ-3’), pMM20 (γAla²⁵⁰⁻²⁵⁰Val²⁵⁰, γ-6’6’), pMM17 (γAla²⁵⁰⁻²⁵⁰Val²⁵⁰, γ-9’9’), pMM8 (γAla²⁵⁰⁻²⁵⁰Val²⁵⁰, γ-12’), pMM18 (γAla²⁵⁰⁻²⁵⁰Val²⁵⁰, γ-15’), and pMM19 (γAla²⁵⁰⁻²⁵⁰Val²⁵⁰, γ-18’). All mutations were confirmed by sequencing (27). The resultant plasmids were transformed into E. coli strain DS8 (28), which contains an uncB-uncC deletion and hence has lost the ability to functionally express FₒF₁-ATPase. The mutants were tested for expression of a functional ATP synthase by growth on solid succinate medium (34 mM KH₂PO₄, 64 mM KH₂PO₄, 0.3 mM MgSO₄, 20 mM (NH₄)₂SO₄, 1 μM FeSO₄, 1 μM ZnCl₂, 10 μM CaCl₂, 50 μM 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). Fore the Ribi press passage. Instead the buffer for resuspension of the ampicillin model was added to a final concentration of 100 μg/ml. Cells were harvested at A₆₀₀ = 0.8. Bacterial preparation and EF isolation were carried out essentially as described previously (29). Cells were not treated with phenylmethylsulfonyl fluoride and pancreatic DNase before the Brie press passage. Instead the buffer for resuspension of the cells was supplemented with an EDTA-free mercaptate inhibitor mixture (Roche Diagnostics, Mannheim, Germany, 1 tablet/100 ml). The crude supernatant of the final wash step (containing the Fₒ-ATPase) was applied to an anion exchange column (Tosoh Fractogel TSK-DEAE 560S). Toyo Soda, Darmstadt, Germany) equilibrated with buffer A (50 mM Tris/H₂SO₄, 10% (v/v) methanol, pH 8.3). Using a stepwise salt elution by addition of buffer B (buffer A + 0.5 M Na₂SO₄), EF₅F₄F₃F₂F₁ was eluted in the 75–150 mM Na₂SO₄ fraction (10–20 mg protein/12 liters of culture volume). After addition of 1 mM MgATP the protein was precipitated with 3.2 mM (NH₄)₂SO₄ and stored at 4 °C.

**Rotation Assay**—2–3 μg protein were purified from the (NH₄)₂SO₄ precipitate by gel filtration through NAP-10 columns (Amersham Bio- sciences, Freiburg, Germany), which were equilibrated with 20 mM MOPS-KOH, 5 mM MgCl₂, 50 mM KCl, pH 7.0 (buffer C). After de-termination of the protein concentration the 20-μl molar excess of Biotin-PEAC₅-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 EF₅ 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-Glas-top/di in Culture plastic). All of the 4 ml of the incubation chamber was filled with buffer D (g/ml thiamine, 0.12 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.
... A D N G G S L I K E L Q L V Y N K A
pKKh7 5'-GCAGCACTCAATGGCGAGCTGTTAATAAACAGGCTGATATCAACAAAAAGCT
pMM16 5'-GCAGCACTCAATGGCGAGCTGTTAATAAACAGGCTGATATCAACAAAAAGCT
pMM20 5'-GCAGCACTCAATGGCGAGCTGTTAATAAACAGGCTGATATCAACAAAAAGCT
pMM17 5'-GCAGCACTCAATGGCGAGCTGTTAATAAACAGGCTGATATCAACAAAAAGCT
pMM19 5'-GCAGCACTCAATGGCGAGCTGTTAATAAACAGGCTGATATCAACAAAAAGCT

β-start R G S H H H H H H G...

RESULTS AND DISCUSSION

Construction of uncG Deletion Mutants and Purification of EF1(γ-x)—Aiming at the function of the C terminus of ATP synthase subunit γ, we constructed six mutants of E. coli F1F0-ATPase containing C-terminally truncated γ subunits (γ-3, -6, -9, -12, -15, and -18) by substitution of the KpnI/SacI fragment of pKKh7 (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 EF1(γ-15) and EF1(γ-18), the complexes were assembled but that they became increasingly unstable. A slight increase of the doubling times (EF1-KH7 (control) → EF1(γ-12)) paralleled a decrease of the F1-ATPase activities of solubilized EF1(γ-x). Prolonged doubling times of EF1(γ-15) and EF1(γ-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 EFγEF1. These cells had to rely on glycolysis for the synthesis of ATP, just as E. coli strain DK8 without any plasmid. In comparison with EF1(γ-15) and EF1(γ-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 EF1(γ-15) and EF1(γ-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 EF1 preparations was checked by SDS-polyacrylamide gel electrophoresis (Fig. 3). It was evident that all EF1 preparations had the same α:β:γ ratio. The ATP hydrolysis activities of the purified EF1(γ-αx) at 35 °C decreased with increasing deletion length (Table I). EF1(γ-3) showed 70%, EF1(γ-6) 50%, EF1(γ-9) 30%, and EF1(γ-12) 24% of the hydrolysis activity of the control (EF1-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 EF1(γ-4) and EF1(γ-10), respectively. EF1(γ-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 EF1 (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 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 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 EF1(γ-4) and EF1(γ-10), respectively. EF1(γ-18) in their hands also was not capable of growing by oxidative phosphorylation (15).

\[
T = \frac{4\pi}{3} \left( \frac{2\pi rL^3}{\ln \left( \frac{L}{r} \right) - 0.447} \right)
\]

where \(T\) denotes the torque, \(r\) the rotational rate, \(\gamma\) 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...
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TABLE I

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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 lane contains 0.3 µg of protein.

viscous drag of the bulk medium as determined by the viscosity of bulk water, namely η(H₂O) = 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 nm²/s³, 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 EF1(γ-x) and in the control. The line shows the rate expected for a torque of 51 pN nm. Within

Fig. 4. Comparison of the maximal average torques generated by EF1-KH7 and EF1(γ-x) during rotation. The average torque of the control (51 pN nm) was set to 100%. The decrease of the average torques in EF1(γ-x) was not significant.

Fig. 5. Rotational rate dependent on filament length for EF1-KH7 and EF1(γ-x). The “isotorque” at 51 pN nm was calculated with an apparent surface viscosity η = 2.4·10⁻³ kg/(m s). 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).
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-phalloidin-labeled actin filaments. The perpetuation of rotation did not significantly differ between EF1(γ-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 (EF1-KH7) and EF1(γ-x). The decreased ATPase activity of the truncation mutants obviously was not caused by more frequent lapses into the Mg-ADP-inhibited state.

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 $V_{\text{max}}$, 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 EF1 (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 EF1(γ-12) and EF1(γ-9) were almost linear over the entire temperature range (with a minor decrease at temperatures above 35 °C), the hydrolysis activities of the control (EF1-KH7) and of EF1(γ-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, EF1(γ-x), showed lower activation energy (cf. Table I) than the control, e.g. 34 kJ/mol (EF1(γ-12)) as compared with 54 kJ/mol (EF1-KH7). This was consistent with, but did not prove, a weakened interaction of the C terminus of γ with (αβ)$_{10}$. 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 EF1(γ-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. ten-toxin sensitivity to the spinach enzyme (40). These portions of chloroplast α and β may increase the interactions between (αβ)$_{10}$ and γ in CF1 to an extent even allowing for a 20-residue truncation to be tolerated. In contrast in EF1 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 (αβ)$_{10}$ 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 EF1-KH7 and EF1(γ-3) crossed over those of EF1(γ-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 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 (αβ)$_{10}$ 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 EF₃ 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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REFERENCES