F1-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 F1-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 (Escherichia coli F1-ATPase). The ATP hydrolysis activity of a load-free ensemble of F1 with 12 residues deleted decreased to 24% of the control. EF1, with deletions of 15 or 18 residues was inactive, probably because it failed to assemble. The average torque generated by a single molecule of EF1, 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 EF1 (full-length γ) to 34 kJ/mol for EF1(γ-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 F0F1-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 F0 part translocates protons, thereby converting protonotive force into the mechanical energy of rotary motion. Rotation is forwarded through the shaft into the F1 part where it drives ATP synthesis. In ATP hydrolysis mode the rotation is reversed, and ions are pumped through F0 in the opposite direction. The Escherichia coli enzyme (EF1),† has the simplest subunit composition. It consists of eight different subunits, five in the peripheral F1 portion and three in the membrane-intrinsic F0, with stoichiometries of (αβ)3γδε for F1 and probably αβεc10 for F0 (12). In view of the rotary mechanism they also can be organized into “rotor” (γεδ) and “stator” (αβab). According to the crystal structure of bovine heart mitochondrial F1 (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)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) EF1 with truncated γ (lacking 10 C-terminal residues) was still active (15). 2) The ATPase activity of the homologous enzyme from chloroplasts (CF1) 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 promotive 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 (Thr277→Val286 (21)). 6) Most surprisingly, a covalent link between the penultimate C-terminal residue of EF1-γ and a nearby residue of α(γA285C ↔ αP280C (22)) neither inhibited ATP hydrolysis nor the rotation of subunit γ relative to (αβ)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 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...
鲢 effect on the functioning of assembled enzyme molecules, their activation barrier was determined by a mechanical constraint. Truncated constructs decreased from 54 to 34 kJ/mol, as if the freely running enzyme, however, the activation energy of the enzyme the torque remained the same as in controls. In the function of the enzyme. During rotation phases of the loaded micro-videographic rotation assay (10) F1 works against the viscous drag of an actin filament of micrometer length. Under these conditions, the F1-ATPase operates un-

FIG. 1. Schematic representation of E. coli F1. Two copies each of subunit 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 F1. Since a mechanical load is absent under these conditions, the F1-ATPase operates under kinetic control. In the holoenzyme, F0-F1, on the other hand, F1 works against the ion-driven F0 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 turnover 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), MB 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-nitrotriacetic acid (Ni-NTA) superflow and nickel-nitrotriacetic 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 cysteine substituted by Ala (24), Hisα-tag extension at the N terminus of subunit β, yK109C (25) plasmid pMM4 was generated by transferring the KpnI/SacI fragment of pKH7 (containing the entire coding region of uncG and part of the sequence of uncD (Ala2–Leu182) into pBlueScript II SK +/–. Based upon a method described by Weiner et al. (26), deletions were performed in uncG by PCR with the following 5′ phosphorylated oligonucleotides as 3′-5′-primers: 3′-5′-GAGCCCCCGAGACGATCTCG-GTGAAGTCCTG-3′, 5′-GAGCAGATTCCGGTATCTGAGTAAAGGAT-GCTGG-3′, 5′-GGTGAAGTCCTGAGTAAAGGAT-GCTGG-3′, 5′-GCTGAATGCTGGAGTGAACGATCTCG-GTGAAGTCCTG-3′, 5′-GCTGGAGTGAACGATCTCG-GTGAAGTCCTG-3′, 5′-GCTGAATGCTGGAGTGAACGATCTCG-GTGAAGTCCTG-3′, 5′-GCTGGAGTGAACGATCTCG-GTGAAGTCCTG-3′, 5′-GCTGAATGCTGGAGTGAACGATCTCG-GTGAAGTCCTG-3′, 5′-GCTGGAGTGAACGATCTCG-GTGAAGTCCTG-3′, 5′-GCTGAATGCTGGAGTGAACGATCTCG-GTGAAGTCCTG-3′, and 3′-5′-primer the 5′-phosphorylated oligonucleotide 5′-P-TAAACAGGTTATTTCGTAGAGGAT-TTACCAACTCGACGTC-3′, and as 5′-3′-primer the 5′-phosphorylated oligonucleotide 5′-P-TAAACAGGTTATTTCGTAGAGGAT-TTACCAACTCGACGTC-3′, and as 5′-3′-primer the 5′-phosphorylated oligonucleotide 5′-P-TAAACAGGTTATTTCGTAGAGGAT-TTACCAACTCGACGTC-3′, and as 5′-3′-primer the 5′-phosphorylated oligonucleotide 5′-P-TAAACAGGTTATTTCGTAGAGGAT-TTACCAACTCGACGTC-3′, and as 5′-3′-primer the 5′-phosphorylated oligonucleotide 5′-P-TAAACAGGTTATTTCGTAGAGGAT-TTACCAACTCGACGTC-3′, and as 5′-3′-primer the 5′-phosphorylated oligonucleotide 5′-P-TAAACAGGTTATTTCGTAGAGGAT-TTACCAACTCGACGTC-3′, and as 5′-3′-primer the 5′-phosphorylated oligonucleotide 5′-P-TAAACAGGTTATTTCGTAGAGGAT-TTACCAACTCGACGTC-3′, and as 5′-3′-primer the 5′-phosphorylated oligonucleotide 5′-P-TAAACAGGTTATTTCGTAGAGGAT-TTACCAACTCGACGTC-3′, and as 5′-3′-primer the 5′-phosphorylated oligonucleotide 5′-P-TAAACAGGTTATTTCGTAGAGGAT-TTACCAACTCGACGTC-3′, and as 5′-3′-primer the 5′-phosphorylated oligonucleotide 5′-P-TAAACAGGTTATTTCGTAGAGGAT-TTACCAACTCGACGTC-3′, and as 5′-3′-primer the 5′-phosphorylated oligonucleotide 5′-P-TAAACAGGTTATTTCGTAGAGGAT-TTACCAACTCGACGTC-3′, and as 5′-3′-primer the 5′-phosphor...
microscope (IX70, Olympus, Japan; lens PlanApo 100×/1.40 oil, fluorescence cube MWIG) and recorded with a VHIS-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-biotinylated F-actin) prevented the binding of fluorescent F-actin, as evident from the absence of fluorescent filaments within 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 MgCl₂, 5 mM KCN, 2 mM phenylmethylsulfonyl fluoride, 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 EFγ. The decrease of NADH absorption at 340 nm was recorded for 5–30 min.

Other Procedures—Polyacrylamide 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 EFγ(γ-x)—Aiming at the function of the C terminus of ATP synthase subunit γ, we constructed six mutants of E. coli F₁,Fₐ-Pase containing C-terminally truncated γ subunits (γ-3, -6, -9, -12, -15, and -18) by substitution of the KpnI/SacI 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), pMM18 (γ-12), pMM19 (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 EFγ(γ-15) and EFγ(γ-18), the complexes were assembled but that they became increasingly unstable. A slight increase of the doubling times (EFγ-KH7 (control) → EFγ(γ-12)) paralleled a decrease of the F₁,Fₐ-Pase activities of solubilized EFγ(γ-x). Prolonged doubling times of EFγ(γ-15) and EFγ(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 EFγEFγ. These cells had to rely on glycolysis for the synthesis of ATP, just as E. coli strain DK8 without any plasmid. In comparison with EFγ(γ-15) and EFγ(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 EFγ(γ-15) and EFγ(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 EFγ preparations was checked by SDS-polyacrylamide gel electrophoresis (Fig. 3). It was evident that all EFγ preparations had the same α:β:γ ratio. The ATP hydrolysis activities of the purified EFγ(γ-x) at 35 °C decreased with increasing deletion length (Table I). EFγ(γ-3) showed 70%, EFγ(γ-6) 50%, EFγ(γ-9) 30%, and EFγ(γ-12) 24% of the hydrolysis activity of the control (EFγ-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 EFγ(γ-4) and EFγ(γ-10), respectively. EFγ(γ-18) in their hands also was not capable of growing by oxidative phosphorylation (15).

Effect of Deletions at the C Terminal of γ on the Torque of EFγ (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 observed with EFγ-KH7, 15 with EFγ(γ-3), 6 with EFγ(γ-6), 2 with EFγ(γ-9), and just 1 with EFγ(γ-12).

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

\[
T = \frac{4\pi}{3} \left( \frac{2\eta v L^2}{L^2 - 0.447} \right) \quad \text{(Eq. 1)}
\]

where \(T\) denotes the torque, \(v\) 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
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 overnight 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 an initial concentration of 10 µg/ml. 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. Growth, doubling times, yields, ATPase activities, and activation energies of EF1-KH7 and EF1(γ-x) were calculated from the slopes of the plots shown in Fig. 6 for the range between 20 and 35 °C.

Growth, doubling times, yields, ATPase activities, and activation energies of EF1-KH7 and EF1(γ-x)

| 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 | EF1 yield per liter glycerol-containing minimal medium | Hydrolysis activity of P1 at 35 °C, pH 8 | Activation energy (Ea) at 30 °C |
|--------------------------------------------------|------------------------------------------|---------------------------------------------------------------|--------------------------------------------------|--------------------------------------------|-----------------------------|
| pH7 (control)                                    |                                          |                                                                |                                                  |                                            |                             |
| pMM16 (γ-3)                                      | −                                        | 139 ± 6                                                       | 1.5 ± 0.3                                        | 93 ± 8                                     | 54 ± 8                       |
| pMM20 (γ-6)                                      | +                                        | 145 ± 11                                                     | 1.5                                              | 65 ± 6                                     | 41 ± 6                       |
| pMM17 (γ-9)                                      | +                                        | 153 ± 8                                                      | 1.2                                              | 46 ± 4                                     | 36 ± 5                       |
| pMM8 (γ-12)                                      | +                                        | 156 ± 4                                                      | 1.2                                              | 28 ± 2                                     | 35 ± 5                       |
| pMM18 (γ-15)                                     | +                                        | 157 ± 10                                                     | 0.8 ± 0.2                                        | 22 ± 2                                     | 34 ± 5                       |
| pMM19 (γ-18)                                     |                                           | 202                                                           |                                                   |                                            |                             |

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/s/m³, 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 handler 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 (∆G = 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/f/(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).
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 (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 Vmax, 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)n. 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 a and β conferring, e.g. ten-toxin sensitivity to the spinach enzyme (40). These portions of chloroplast a and β may increase the interactions between (αβ)n 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)n 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 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 (αβ)n 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 EF₁ 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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