

Domain III of Elongation Factor G from *Thermus thermophilus* Is Essential for Induction of GTP Hydrolysis on the Ribosome*

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Two elongation factors (EF) EF-Tu and EF-G participate in the elongation phase during protein biosynthesis on the ribosome. Their functional cycles depend on GTP binding and its hydrolysis. The EF-Tu complexed with GTP and aminoacyl-tRNA delivers tRNA to the ribosome, whereas EF-G stimulates translocation, a process in which tRNA and mRNA movements occur in the ribosome. In the present paper we report that: (a) intrinsic GTPase activity of EF-G is influenced by excision of its domain III; (b) the EF-G lacking domain III has a 10³-fold decreased GTPase activity on the ribosome, whereas its affinity for GTP is slightly decreased; and (c) the truncated EF-G does not stimulate translocation despite the physical presence of domain IV, which is also very important for translocation. By contrast, the interactions of the truncated factor with GDP and fusidic acid-dependent binding of EF-G-GDP complex to the ribosome are not influenced. These findings indicate an essential contribution of domain III to activation of GTP hydrolysis. These results also suggest conformational changes of the EF-G molecule in the course of its interaction with the ribosome that might be induced by GTP binding and hydrolysis.

The process of protein biosynthesis by the ribosome includes three main phases: initiation, elongation, and termination. Some of translation factors catalyzing individual steps in all these phases are GTP/GDP-dependent proteins (1). While complexed with GTP they bind to the ribosome and after GTP hydrolysis dissociate as GDP-bound inactive proteins. Elongation factors (EFs)¹ Tu and G are involved in the elongation phase of protein biosynthesis. The tertiary structure of EF-Tu from *Thermus aquaticus* in both GTP and GDP forms are known at 2.5 and 2.7 Å resolutions, respectively (2, 3). The structure of the ternary complex of Tu with tRNA, and the uncleavable analog of GTP (GMPPNP) was also determined (4).

The crystallographic studies of EF-G from *Thermus thermophilus* resulted in determination of the EF-G structure in complex with GDP and its empty form (5, 6). These studies revealed structural similarities (“molecular mimicry”) between tertiary structures of EF-G and the EF-Tu-aminoacyl-tRNA

ternary complex (7, 8). It is interesting that domains III, IV, and V of EF-G mimic the tRNA structure in the EF-Tu ternary complex, whereas domain IV can be related to the tRNA anticodon arm. The G domains in both factors are nucleotide-binding domains.

These findings encouraged the studies of the molecular mechanism of EF-G interaction with the ribosome and the functional role of its domains. It was reported that EF-G lacking domain V was deficient in nucleotide-dependent association with the ribosome (9). Biochemical studies have also shown that EF-G domain IV (10, 11) and, in particular, the intact conformation of its distal end (12) are very important for translocation in the ribosome, whereas GTPase activity and nucleotide-dependent ribosome association of factor G are unaffected by domain IV excision.

To further study the functional role of EF-G domains, we have obtained a truncated variant of *T. thermophilus* EF-G lacking domain III (EF-GΔIII). The effects of EF-GΔIII on (a) GTP hydrolysis, (b) its interaction with nucleotides and fusidic acid, and (c) stimulation of translocation were studied. It was found that the rate of GTP hydrolysis catalyzed by EF-GΔIII was markedly decreased, and the mutated protein was unable to promote translocation, whereas the affinity of EF-GΔIII to GDP and its interaction with fusidic acid in the presence of ribosomes were not affected. These results are discussed in the light of structural rearrangements in the EF-G and the ribosome upon their interaction.

EXPERIMENTAL PROCEDURES

Reagents—Restriction endonucleases and T4 DNA ligase were from Promega. High fidelity *Pfu* Turbo DNA polymerase was purchased from Stratagene and used according to the manufacturer’s manual. DEAE-Sepharose FF and Phenyl-Sepharose were from Amersham Pharmacia Biotech, HA-Ultrogel was from Sigma. Plasmids were maintained in *Escherichia coli* strain XL1 (Promega). For gene expression plasmid pET11c and strain B834(DE3) (Novagen) were used. The polyethyleneimine cellulose sheets for thin layer chromatography were from Merck.

Oligonucleotides and Mutagenesis—The following oligonucleotides were synthesized by Gene Assembler Plus (Amersham Pharmacia Biotech) according to the manufacturer’s manual: Pr1, 5′-**CCCGTGATTGACCC**CCAGGTGGCGTACCGGGA-3′; Pr2, 5′-**CGCCACCTGGGG**-GTCAATCACGGGCTCGGGC-3′; Pr3, 5′-CGGTGGTGCATATGGCGG-TCAAGGTAG-3′; and Pr4, 5′-GCGAATTCTATTGACCCTTGATGAGC-3′. The oligonucleotides Pr1 and Pr2 are designed so that their 3′ ends hybridize to template sequence on one side of the deletion, and the 5′ ends (given in bold letters) are complementary to the template DNA on the other side of the deletion. The primers Pr3 (with *Nde*I recognition site and ATG codon) and Pr4 containing *Eco*RI site and TAA stop codon are the flanking primers. The recognition sites of endonucleases are underlined.

The DNA fragment coding domain III was deleted according to the splicing overlap extension technique (13). All other recombinant DNA procedures were performed according to the published manual (14).

Plasmid pLS3 carrying the *fus* gene from *T. thermophilus* was used as a template (15). At the first step two independent PCR rounds were performed with primers Pr1 and Pr4 or primers Pr2 and Pr3. The reactions were carried out for 20 cycles of denaturation (1 min at 94 °C),

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¹ The abbreviations used are: EF, elongation factor; GMPPNP, guanosine 5′-[β,γ-imido]triphosphate; wt, wild type; EF-GΔIII, elongation factor lacking domain III; PCR, polymerase chain reaction.

annealing (30 s at 58 °C), and extension for 90 s at 72 °C. At the next step, two purified DNA fragments from the first PCR round were mixed and subjected to splicing by PCR for 15 cycles of denaturation (1 min at 94 °C), annealing (30 s at 65 °C), and elongation (150 s at 72 °C). Reactions were performed in the buffer supplied with DNA polymerase. The reaction mixture (100 μ l) contained 100 μ mol of dNTPs; 250 ng of pLS3 DNA, 1 μ M primers (or 200–300 ng/50- μ l DNA fragments obtained in the first PCR), and 2.0 units of *Pfu* Turbo DNA polymerase.

The product from the second PCR was treated with *Nde*I and *Eco*RI endonucleases and cloned into plasmid pET11c cleaved with the corresponding nucleases. As a result, plasmid pET Δ III containing the *fus* gene with a deletion of nucleotides 1231–1434 was obtained, and mutation was confirmed by sequencing.

Protein Production and Purification—The *E. coli* B834(DE3) strain was used for gene expression in the pET expression system (16). Cell growth and protein isolation were performed according to the published procedure (17). According to electrophoresis (18), the purified protein was homogeneous. The concentration was determined photometrically, taking $A_{280} = 0.6$ for 1 mg/ml of *T. thermophilus* EF-G.

Nucleotide Binding—GDP interactions with EF-G and dissociation constant were tested according to the published procedure (19). The reaction mixture (0.2 ml) in 50 mM Tris-HCl buffer, pH 7.6, 10 mM MgCl₂, 1 mM dithiothreitol, 70 mM NH₄Cl contained 300 pmol of EF-G and varied amounts of [³H]GDP (1.1 Ci/mmol) at 37 °C were used.

The affinity of the mutated protein to GMPPNP was measured by displacement of [³H]GDP bound to the factor (300 pmol) with unlabeled GMPPNP. The concentration of [³H]GDP in the mixture was 0.5 μ M, and that of GMPPNP varied from 0.5 to 500 μ M.

Nucleotide-dependent formation of EF-G complexes with the ribosomes was monitored by nitrocellulose filtration. The reaction mixture (100 μ l) in 50 mM Tris-HCl buffer, pH 7.6, 10 mM MgCl₂, 1 mM dithiothreitol, 20 mM NH₄Cl contained 26 μ M [³H]GMPPNP (950 mCi/mmol), 50 pmol of 70 S ribosomes, and 150 pmol of EF-G. After nucleotide addition the mixture was incubated at 37 °C for 15 min and then cooled on ice. An aliquot of 40 μ l was filtered, and the filter was washed once with 2.5 ml of ice-cold incubation buffer. The radioactivity was measured in a scintillation counter.

A similar procedure was used to assay the EF-G-Rs-GDP complex formation, except that either 17.8 μ M [³H]GDP (110 mCi/mmol) or 7.3 μ M [³H]GTP (8.1 Ci/mmol) was used. Incubation was carried out either on ice or at 37 °C for 10 min, the reaction mixture was diluted with 950 μ l buffer, 250- μ l aliquots were rapidly filtered through a nitrocellulose filter, and the filter was washed with 2.5 ml of ice-cold reaction buffer. In parallel experiments fusidic acid at 1 mM concentration was used to follow the stabilization of EF-G-GDP binding to the ribosome.

GTPase Activity—The assay of ribosome-dependent GTPase activity of EF-G was performed in 10 μ l of reaction mixture in the 50 mM Tris-HCl buffer, pH 7.6, 10 mM MgCl₂, 70 mM NH₄Cl, 1 mM dithiothreitol, containing 82.5 μ M [³H]GTP (2 Ci/mmol), 10 pmol of *E. coli* 70 S ribosomes, and either 1 or 150 pmol of wt EF-G or EF-G Δ III, respectively, at 37 °C.

The ribosome independent (intrinsic) GTPase activity of the factors was checked in the mixture (10 μ l) containing 2.4 μ M [³H]GTP (8.1 Ci/mmol) and 150 pmol of EF-G at 55 °C. Aliquots (2 μ l) were taken periodically, mixed with 0.5 μ l 10 mM GDP/GTP solution in 8% formic acid and placed onto polyethyleneimine-cellulose sheets for TLC (20). The nucleotides spots were visualized under UV light, excised, and placed into scintillation liquid for [³H]GDP counting.

Kinetic Parameters of GTPase—The determination of kinetic parameters of GTPase reaction were carried out at 37 °C at 1 μ M 70 S ribosomes, with 0.5 pmol of wt EF-G or 60 pmol of EF-G Δ III and varied concentration (10–200 μ M) of [³H]GTP (4.05 Ci/mmol). Alternately, the same quantities of the factors were incubated for with 38 μ M [³H]GTP (8.1 Ci/mmol) and varied concentration of the 70 S ribosomes (0.03–0.64 μ M). Incubation time was 3 and 15 min for wt and the truncated factors, respectively.

The reaction mixture with 6.6 pmol of wt EF-G (or 126 pmol of EF-G Δ III) incubated for 20 min (60 min for EF-G Δ III) served for determination kinetic constants of intrinsic GTPase at 55 °C. Eadie-Hofstee plots were used to determine V_{max} and K_m values. The V_{max} values, reaction time, and EF-G amount stated above were used to calculate k_{cat} .

Translation and Translocation Assays—Poly(U)-directed polyphenylalanine synthesis was carried out in a cell-free translation system with purified elongation factors Tu and G according to the published procedure (21).

Translocation activities of mutated and wt factors were also checked by competence of the charged ribosomal complex to puromycin. In this

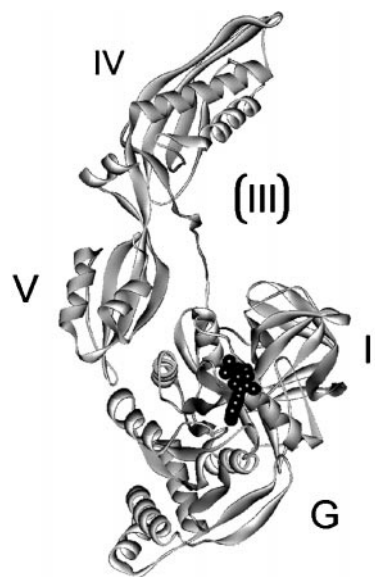


FIG. 1. Ribbon model of the tertiary structure of *T. thermophilus* EF-G lacking domain III. The contour of GDP molecule is shown in the G domain. The position of deleted domain III indicated by roman figure in brackets. The model was drawn using Protein Data Bank coordinates (code 1DAR) supplied by *Ævarsson et al.* (6).

case, the pretranslocation complex was prepared as described (22) and contained 34 pmol of [¹⁴C]Phe-tRNA^{Phe} bound to the A-site of 45 pmol ribosomes. The puromycin reaction (final volume, 125 μ l) was carried out at 1 mM puromycin and 40 pmol of wt EF-G (or 100–300 pmol of EF-G Δ III) added to the charged ribosomes (23). The Phe-puromycin formed in the reaction was extracted with ethyl acetate, and its radioactivity was counted.

All experiments described in the above sections were repeated at least twice.

RESULTS

Mutagenesis and Protein Isolation—The EF-G molecule contains five distinct domains (5, 6). The N-terminal domain (G domain) is a nucleotide-binding domain. All other domains are numbered consecutively along the amino acid sequence. Domain III makes covalent connection between domains II and IV. To prepare the deletion mutant of *T. thermophilus*, DNA triplets 411–478 encoding domain III were excised from the *fus* gene by the PCR technique (see “Experimental Procedures”). As a result the amino acid residues 403–410 and 479–483 linked by a new peptide bond between Asp⁴¹⁰ and Pro⁴⁷⁹ connect now domains II and IV (Fig. 1). EF-G Δ III contains 623 amino acid residues and has the molecular mass of 69.4 kDa.

The mutated protein was produced in a soluble form in the *E. coli* expression system. Because the mutant remained highly thermostable, a heat denaturation step was used during its isolation as it was done earlier for the wild type and mutated EF-G (15, 17). These results indicate that the truncated protein is correctly folded. The yield of the purified protein was 7 mg from 5 g of the wet cells.

Nucleotide Interactions—The wild type and mutated proteins form strong binary complexes with GDP with the dissociation constant of 1.5 μ M. This K_d value is close to the dissociation constant (0.67 μ M) reported for the wild type EF-G from *T. thermophilus* (19). Both binary complexes bind to ribosomes and compose a relatively strong ternary complex (Tables I). These results also indicate that EF-G Δ III was correctly folded.

However, EF-G Δ III with an uncleavable GTP analog (GMPPNP) binds less tightly to the ribosome than wt EF-G. The filter binding technique revealed that only 2.4 pmol of truncated factor and GMPPNP was bound to the ribosomes, whereas the corresponding value for intact EF-G was 21.8

TABLE I

GDP-dependent formation of EF-G complexes with ribosomes

Yields are given in pmol/50 pmol of ribosomes in the presence of 150 pmol of the factors and averaged from three independent experiments.

Components in the mixture	Complexes	
	With EF-GΔIII	With EF-G
	<i>pmol</i>	
EF-G, GDP, Rs	21.5	17.9
EF-G, GDP, Rs, FA	31.8	35.4
EF-G, GTP, Rs, FA	3	48
EF-G, GTP, Rs, FA ^a	23.6	50.3

^a The reaction was carried out at 37 °C and in all other cases at 0 °C.

pmol. Dissociation constants determined for the binary complex with GMPPNP were 240 μM for EF-GΔIII and 94 μM for intact EF-G (Table II). These data show that nucleoside triphosphate association with EF-GΔIII is somewhat impaired both in the absence and presence of ribosomes.

GTPase Activities—Intact EF-G has intrinsic GTPase activity at 55 °C. This activity was affected by domain III excision, and the k_{cat} value for EF-GΔIII was 43 times lower than that of wt EF-G (Table II). The K_m values for the intact and truncated factors were 20 and 90 μM, respectively. Hence, a decreased intrinsic GTPase activity of EF-GΔIII indicates that excision of domain III influences the nucleotide-binding center located in G domain of the factor (5, 24). On the other hand, whereas intrinsic GTPase activity of wt EF-G was greatly stimulated by the 70 S ribosome, only slight stimulation was observed in the case of a truncated factor. The turnover of GTP hydrolysis catalyzed by EF-GΔIII at 37 °C in the presence of ribosomes was much lower than in the case of intact EF-G (Fig. 2).

In the case of ribosome-dependent GTP hydrolysis, the kinetic measurements gave comparable K_m values for the mutated (40 μM) and intact proteins (25 μM) but quite different catalytic constants (Table II). In the presence of ribosomes, the ratio $0.63/6.7 \times 10^{-4}$ of k_{cat} values for intact and mutated factors is equal to 940. This result means that catalytic activity of the truncated factor is greatly decreased.

Translation and Translocation Activities—It was reported that wt EF-G from *T. thermophilus* was active in poly(U)-directed translation with *E. coli* ribosomes (15, 17). Intact EF-G promotes translocation by catalyzing formation more than 35 pmol of Phe-puromycin in the reaction with the charged ribosomes for 15 min (Fig. 3). On the other hand, the truncated protein was not active either in translation or in translocation tests even at 2–3 molar of its excess. The yield of Phe-puromycin reaction in the presence of EF-GΔIII was equal to that in the control experiments without EF-G addition (Fig. 3), *i.e.* the truncated factor did not increase the level of spontaneous translocation revealed in the factor-free system (25). These results show that specific GTPase activity of the truncated factor is not sufficient to ensure a normal translocation event in the ribosome.

Fusidic Acid Interactions—Fusidic acid stabilizes ribosomal complexes both with the intact and truncated factors when GDP is present in the reaction mixture (Table I). The complex is also formed in the presence of GTP. However, in this case the complex formation with the truncated factor was temperature-dependent. 3 pmol of the complex with EF-GΔIII and fusidic acid was formed at 0 °C for 15 min, and 23.6 pmol at 37 °C, whereas corresponding values at 0 °C and 37 °C for the intact factor were virtually equal (48 and 50.3 pmol respectively, Table I). These results correlate with the decreased GTPase activity of the truncated factor (Fig. 2) and indicate that binding of fusidic acid occurs after GTP hydrolysis. These findings agree with the mechanism proposed for the uncoupled GTP hydrolysis catalyzed by *E. coli* EF-G (26).

TABLE II

Kinetic parameters for the intact and truncated factors

Parameter ^a	EF-GΔIII	EF-G
K_d (GDP), μM	1.5 ± 0.3	1.5 ± 0.3
K_d (GMPPNP), μM	240 ± 10	94 ± 4
For Rs-dependent GTPase		
K_m (GTP), μM	40 ± 5	25 ± 3
K_m (Rs), μM	0.25 ± 0.02	0.18 ± 0.02
k_{cat} (s ⁻¹)	6.7 ± 1 × 10 ⁻⁴	0.63 ± 0.2
For intrinsic GTPase		
K_m (GTP), μM	90 ± 5	20 ± 3
k_{cat} (s ⁻¹)	1.5 ± 0.1 × 10 ⁻⁴	64 ± 10 × 10 ⁻⁴

^a At least two independent experiments were carried out to determine the given values. 300 pmol of the factors and varied amount of nucleotides were used for K_d determination. Experimental details for the determination of the kinetic parameters of Rs-dependent and intrinsic GTPase reactions are given under "Experimental Procedures."

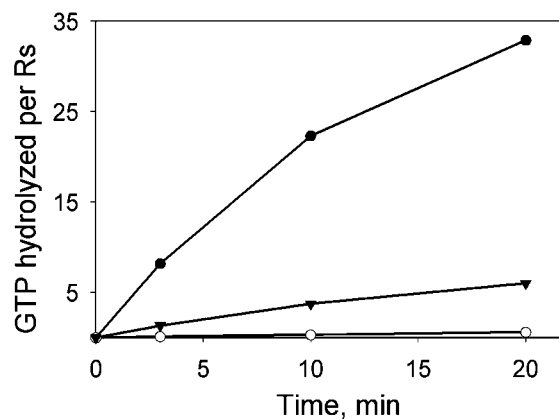


FIG. 2. The turnover of GTP hydrolysis on the ribosomes catalyzed by EF-G and EF-GΔIII. ●, incubation with 1 pmol of wt EF-G; ▼, incubation with 150 pmol of EF-GΔIII; ○, control without EF-G. The ordinate represents the number of GTP molecules hydrolyzed per ribosome at 37 °C.

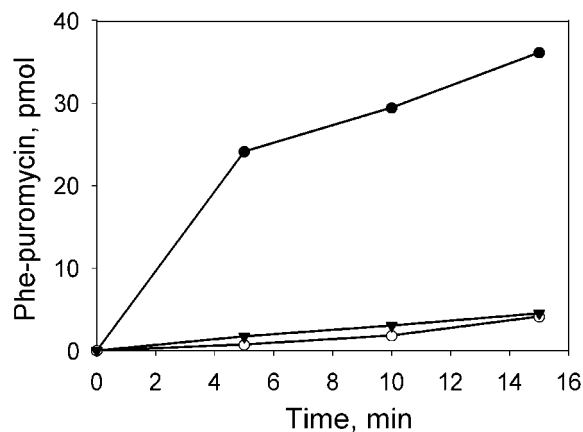


FIG. 3. Kinetics of puromycin reaction with pretranslocation ribosomes promoted by wt EF-G (●) or EF-GΔIII (▼). ○, control without EF-G. 50 pmol of wt EF-G and 100 pmol of EF-GΔIII were used in the reaction with 45 pmol of ribosomes at 37 °C.

DISCUSSION

The results reported above demonstrate that GDP-dependent interactions of the truncated factor are not impaired, both in the absence and in the presence of ribosomes (Table I). This fact may reflect that the GDP bound form of EF-G is a final product of its functional cycle and that domain III is not important for GDP binding.

By contrast, both intrinsic and ribosome-dependent GTPase activity (Fig. 2) of EF-GΔIII was markedly affected (see "Re-

sults"). The GTPase activity of the truncated factor in the presence of ribosomes was 940 times lower than that of wt EF-G, whereas only a 2-fold decrease of its affinity for the ribosome was revealed (Table II). These results mean that low rate of GTPase reaction catalyzed by the truncated factor, compared with wt EF-G, is mainly due to a 10^3 -fold decrease of its catalytic activity rather than to a small decrease in the affinity of binding to the ribosome. Inasmuch as domain III has no direct contact with the nucleotide binding center located on the G domain (5, 24), these findings indicate that domain III influences the GTP-binding center of EF-G as well as interaction of the factor with the ribosome and subsequent induction of GTP hydrolysis. Hence, it can be suggested that conformational changes in EF-G and in the ribosome might occur upon factor binding to the ribosome. Indeed, conformational changes were revealed in the elongation factors (27) and in ribosomal proteins (28, 29) by limited proteolysis of ribosomes complexed with EF-G or EF-Tu in the presence of GDP or GMPPNP, *i.e.* in the complexes modeling post- or pre-hydrolysis states of nucleotide bound to ribosomes.

As stated in the report on interaction of fusidic acid with the ribosome and EF-G, the antibiotic binding occurred after GTP hydrolysis (26). It was also proposed that hydrolysis of the nucleotide might generate or uncover the fusidic acid-binding site (30). The data given in Table I are consistent with this suggestion. It is interesting to note that formation of the ribosomal complex with fusidic acid in the presence of GTP was somewhat increased in comparison with that when GDP was added (Table I). Thus, it seems likely that fusidic acid binding in concert with GTP hydrolysis is more efficient than in the presence of GDP because EF-G may have an intermediate conformational just after GTP hydrolysis that can favor the antibiotic binding.

The tertiary structure of EF-G complexed with GTP is unknown to be compared with the structure of EF-G-GDP complex. Yet, the x-ray scattering studies of nucleotide-free EF-G and its binary complexes with GDP or GTP in solution have not revealed any remarkable structural changes of the molecule, although small scale variations in the EF-G structure were not ruled out, and possible changes in EF-G bound to ribosomes were discussed (31). By contrast, comparison of the EF-Tu-GDP structure (3) with that of EF-Tu-GTP (2, 32) reveals major rearrangement of domains within the EF-Tu molecule in the last complex. These differences between factors G and Tu can be explained by their interactions with guanine nucleotides and ribosomes.

The exchange of GTP for GDP in EF-Tu is catalyzed in the solution or cytoplasm by EF-Ts. On the other hand, no protein analogous to EF-Ts was found for EF-G. However, in the living cell the GTP concentration is 10 times higher than that of GDP (33), and affinity of GTP to EF-G is greatly increased in the presence of ribosome (34). The dissociation constant (59 nM) has been reported for uncleavable GTP analog complexed with *E. coli* EF-G and ribosomes (34). It is over 800 times less than K_d (50 μ M) for the GTP interaction with *E. coli* EF-G without ribosomes (19), and 200 times less than K_d value for GTP (12 μ M) with *T. thermophilus* EF-G in the absence of ribosomes (19). Besides, it should be noted that GDP affinity to EF-G is always lower than affinity to EF-Tu. For example, K_d for GDP complexed with EF-G is 600 times higher than that of the EF-Tu-GDP complex (19). Hence, it is reasonable to believe that particular EF-G features and the conditions in the cell described above are favorable for the GDP/GTP exchange. The binary complex of EF-G with GTP appearing as a result of such an exchange can be readily bound to the ribosome and some adjustments or rearrangements of its domains may be involved

in this process. In any case, the binding of EF-G with GTP (or with its uncleavable analogs) to the ribosome promotes translocation (reviewed in Ref. 35), and after GTP hydrolysis EF-G complexed with GDP dissociates from the ribosome.

As an additional evidence for the conformational changes discussed above, we would like to note that synergism between GTPase activities of EF-Tu and EF-G on ribosomes was revealed, and it was proposed that such positive cooperativity of elongation factors might correlate with different conformations of the ribosome accepting these factors (36). In this connection it is noteworthy that sensitivity of the ribosomal protein L7/L12 to trypsin depends on the elongation factors Tu and G bound to the ribosome, and these results mean that conformational changes may occur in the ribosomal components (29). Conformational changes in the 70 S ribosome and EF-G induced by GTP hydrolysis were recently visualized also by cryo-electron microscopy (37, 38). These findings indicate that after dissociation of a factor, the ribosome is in a state favorable for accepting another.

The data discussed above are also in good agreement with the observation that EF-G domains III, IV, and V mimic the aminoacyl-tRNA moiety of the EF-Tu ternary complex (4, 7, 8). The molecular mimicry of elongation factors implies that dissociation of the EF-G-GDP complex leaves the ribosome being in a state ready for the codon-dependent binding of the EF-Tu ternary complex (39).

In conclusion, the results of this study shows that: (a) both intrinsic and ribosome-dependent GTPase activity of EF-GAIII are significantly decreased; (b) the interaction of the truncated EF-G with GDP as well as fusidic acid-dependent stabilization of the EF-G-GDP complex with the ribosome are not influenced; and (c) the mutated factor is virtually inactive in stimulation of translocation despite the physical presence of domain IV that is also very important for translocation. As discussed above, these results are consistent with conformational changes occurring in elongation factor in accordance with GTP binding and its hydrolysis

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