Regulation of Elongation Factor G GTPase Activity by the Ribosomal State

THE EFFECTS OF INITIATION FACTORS AND DIFFERENTIALLY BOUND tRNA, AMINOACYL-tRNA, AND PEPTIDYL-tRNA

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The elongation factor G (EF-G) is responsible for the translocation of the ribosome along the mRNA chain. Under in vitro conditions, EF-G exhibits a very active uncoupled GTPase activity which is dependent on the presence of ribosomes and is modulated by mRNA-dependent binding of tRNA. In the absence of tRNA, uncoupled EF-G GTPase is inhibited by initiation factors IF1 and IF3, but not by initiation factor IF2. In the presence of N-Met-tRNA^Met and poly(A,U,G) or in the presence of N-acetyl-Phe-tRNA^Phe and poly(U), initiation factor IF2 causes an additional decrease of the uncoupled EF-G GTPase activity. This effect, however, is dependent on the presence of IF1 and IF3 and is obviously due to the mRNA- and initiation factor-dependent binding of N-Met-tRNA^Met and N-acetyl-Phe-tRNA^Phe, respectively, to the ribosomal P-site. Non-enzymatic binding of N-Met-tRNA^Met and N-acetyl-Phe-tRNA^Phe, however, causes a stimulation of uncoupled EF-G GTPase activity. The same effects are observed for Met-tRNA, Phe-tRNA, and uncharged tRNA. These findings are discussed in the light of the three-site model of the ribosome and the mechanism of translocation.

Ribosome-dependent GTP hydrolysis by elongation factor (EF)^G-G is required for the translocation of the ribosome along the mRNA chain (Nishizuka and Lipmann, 1966; Thach and Thach 1971; Gupta et al., 1971; Haselkorn and Rothman-Denes, 1973). Under in vitro conditions, EF-G exhibits a very active uncoupled GTPase activity (Conway and Lipmann, 1964; Nishizuka and Lipmann, 1966; Kaziro et al., 1969). The uncoupling between EF-G GTPase and protein biosynthesis has greatly hampered the determination of a reliable stoichiometry between peptide bond formation and GTP hydrolysis (Nishizuka and Lipmann, 1966). Experimental evidence for the theoretically expected stoichiometry of 2 molecules GTP hydrolyzed per each new peptide bond has been obtained either by utilizing purified, endogenous polysomes and corrected for the uncoupled EF-G GTPase activity (Cabrera et al., 1976) or by using rate limiting concentrations of EF-G (Chinnai and Parmeggiani, 1980).

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‡ The abbreviations used are: EF, elongation factor; IF, initiation factor.

Several years ago, Kuriki and co-workers (Kuriki and Kanno, 1972; Kuriki and Yoshimura, 1974) showed that the ribosomal wash of Escherichia coli contains an inhibitor of uncoupled EF-G GTPase activity. These findings have induced us to study the coupling between EF-G GTPase activity and translocation of the ribosomes by investigating the mechanism by which this inhibitor reduces the uncoupled EF-G GTPase activity. Reinvestigating the results of Kuriki and co-workers (Kuriki and Kanno, 1972; Kuriki and Yoshimura, 1974), we have found two EF-G GTPase inhibitors in the ribosome wash of E. coli. One of these inhibitors has been purified to homogeneity and characterized (Voigt and Nagel, 1990). This factor inhibits both EF-G GTPase and poly(U)-dependent poly(phenylalanine) synthesis, but uncoupled EF-G GTPase activity is considerably more affected (Voigt and Nagel, 1990). Therefore, this inhibitor of uncoupled EF-G GTPase activity has been supposed to be a coupling factor (Voigt and Nagel, 1990). Furthermore, we have found that the 30 S ribosomal subunit is the target site of this inhibitor which consists of two polypeptides with apparent molecular masses of 10,000 and 23,000 (Voigt and Nagel, 1990).Recently, we have provided evidence that this inhibitor of uncoupled EF-G GTPase activity is a complex of initiation factors IF1 and IF3 (Nagel and Voigt, 1992). These findings have induced us to investigate whether or not the EF-G GTPase inhibitor activities are copurified with the initiation factors. Furthermore, we have systematically studied the effects of initiation factors and tRNA (peptidyl-tRNA, aminoacyl-tRNA and uncharged tRNA) on the uncoupled EF-G GTPase activity. The results are discussed on the basis of the recently developed allosteric three-site model for the ribosomal elongation cycle (Rheinberger and Nierhaus, 1986; Hausner et al., 1988; Gnirke et al., 1989).

EXPERIMENTAL PROCEDURES

Materials—ATP (Na^+ salt), GTP (Li^+ salt) and a tRNA mixture from E. coli were purchased from Boehringer (Mannheim, Germany). GTP was passed through a column of AG 50W X-2 cation exchange resin (H^+ form, Bio-Rad). Folinic acid, poly(U), and poly(A,U,G) were supplied by Sigma (Deisenhofen, Germany). DEAE-cellulose (DE-52) and phosphocellulose (P11) were from Whatman (Maidstone, United Kingdom) and Sephadex gels from Pharmacia (Upsala, Sweden). L-[^{35}S]Methionine (specific activity 800 Ci/mmol), L-[^{14}C](U) phosphoethanolamine (specific activity 450 mCi/mmol), and carrier-free 32P were obtained from New England Nuclear (Drumch, Germany). [^{14}C]PCRTP was prepared as recently described (Voigt and Nagel, 1990). All other reagents were analytical grade.

Preparation of Elongation Factors and Ribosomes—Electrophoretically homogeneous elongation factors G (EF-G) and T (EF-T) were prepared from E. coli B (Kaziro and Inoue, 1968; Sander et al., 1975). The EF-G and EF-T preparations used were free of ribosome-independent GTPase activities. Ribosomes, washed two or three times with 0.5 M NH_{4}Cl, were separated into 30 S and 50 S subunits by
sucrose gradient centrifugation at 0.5 mM Mg\(^2\+\) in a Beckman Ti-15 zonal rotor (Sander et al., 1975). Purity and activity of the subunits were tested routinely by analytical sucrose gradient centrifugation and poly(U)-dependent polyphenylalanine synthesis. According to these tests, the 50 S subunits were maximally contaminated with 2-4% 30 S subunits, and the 30 S subunits contained 1-2% functional 50 S subunits. One A,\(\text{subunit}\) of ribosomal particles was taken to represent 25 pmol of 70 S, 39 pmol of 50 S, and 67 pmol of 30 S subunits (Sander et al., 1976). Protein concentrations were measured by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Charging of tRNA—Met-tRNA\(^\text{Met}\) was prepared by charging E. coli MRE 600 tRNA with L-[\(^5\text{S}\)]methionine (specific activity 100 C/\(\mu\)mol) in the presence of \(N'\text{-formyl-tetrahydrofolic acid}\) (folic acid) and a dialyzed E. coli MRE 600 S150 fraction (Dubmoff and Maatra 1971). The tRNA\(^\text{Met}\) was purified to 45% from total tRNA and charged with \(L'-[\(^\text{C}\(\text{U}\)]\)phenylalanine as described by Chimali and Parmegiani (1973). N-Acetyl-Phe-tRNA\(^\text{Pho}\) was prepared by acetylation of \(L'-[\(^\text{C}\(\text{U}\)]\)Phe-tRNA\(^\text{Pho}\) according to Henni and Chapville (1966).

Isolation of EF-G GTPase Inhibitors and Initiation Factors—The ribosome wash from several preparations of ribosomes (corresponding to 1200 g of E. coli B) was combined (21 liters) and adjusted to 65% ammonium sulfate saturation by addition of solid (NFL)\(\text{SO}_4\). After 2 h at 4 °C, the precipitated proteins were collected by centrifugation at 20,000 \(\times\) \(g\) for 20 min. Centrifugation and all of the following purification steps were performed at 4 °C. The precipitated proteins were dissolved in a small volume of 20 mM Tris-HCl (pH 7.5) containing 7 mM 2-mercaptoethanol and subsequently eluted with a linear gradient of 0-100 mM KCl in the same buffer, and subjected to anion exchange chromatography using a Whatman DE-52 column (5.0 \(\times\) 100 cm) eluted with 10 mM Tris-HCl (pH 7.5). The column was washed with 300 ml of buffer A (20 mM Tris-HCl, pH 7.5, containing 7 mM 2-mercaptoethanol) and subsequently eluted with a linear gradient of 0-400 mM KCl in buffer A (2 liters each). Fractions of 20 ml were collected and analyzed for absorbance at 280 nm. Aliquots of 50 \(\mu\)l were taken from every third fraction and, after heating for 10 min at 55 °C, assayed for inhibitors of ribosome-dependent EF-G GTPase as recently described (Voigt and Nagel, 1990). Aliquots from the same fractions were analyzed (without preheating) for the initiation factors IF1, IF2, and IF3, respectively, by measuring the effect on the binding of N-[\(^{5}\text{S}\)]Met-tRNA\(^\text{Met}\) to ribosomes in the presence of poly(A,U,G) and the other two initiation factors as described by Parker Suttle et al. (1973). Two EF-G GTPase inhibitor peaks were eluted from the column as recently described (Nagel and Voigt, 1992). Both IF1 and IF3 activities have been found to be copurified with both EF-G GTPase inhibitors (Fig. 1, lower panel). However, both peaks differ with respect to the relative IF1 and IF3 activity. Whereas the relative activities of EF-G GTPase inhibitor and initiation factors IF1 and IF3, respectively, have been found to be constant in the different fractions of peak I, this is not always true for peak II. A ribosome-independent GTPase activity has been found to be eluted from the DEAE-cellulose column at a salt concentration very similar to that for EF-G GTPase inhibitor peak II (data not shown). In some experiments, there was a partial overlap of the ribosome-independent GTPase activity with the EF-G GTPase inhibitor peak II (data not shown). In some experiments, there was a partial overlap of the ribosome-independent GTPase activity with the EF-G GTPase inhibitor peak II (data not shown). In some experiments, there was a partial overlap of the ribosome-independent GTPase activity with the EF-G GTPase inhibitor peak II (data not shown). In some experiments, there was a partial overlap of the ribosome-independent GTPase activity with the EF-G GTPase inhibitor peak II (data not shown). In some experiments, there was a partial overlap of the ribosome-independent GTPase activity with the EF-G GTPase inhibitor peak II (data not shown).
activity has been found in those fractions containing initiation factor IF2 (Fig. 1).

A complete separation of IF1 and IF3 activities has been observed when peak II eluted from the DEAE-cellulose column (Fig. 1) has been subjected to phosphocellulose chromatography (Fig. 2, lower panel). Both the IF1 and IF3 peak contain EF-G GTPase inhibitor activities (Fig. 2, upper panel). Essentially the same results were obtained when peak I was subjected to phosphocellulose chromatography (data not shown). Subsequent gel exclusion chromatographies of initiation factors IF1 and IF3 on Sephadex G-100 and G-75 columns, respectively, again have revealed copurification of both initiation factors with EF-G GTPase inhibitor activities (data not shown), indicating that the EF-G GTPase inhibitors might be identical with initiation factors IF1 and IF3.

EF-G GTPase is differentially reduced by increasing concentrations of initiation factors IF1 and IF3, respectively (Fig. 3). A considerably more pronounced inhibition of EF-G GTPase activity has been measured in the presence of both initiation factors than in the presence of IF1 or IF3 only (Fig. 3). However, a complete inhibition of EF-G GTPase activity has never been observed even at the highest concentrations of both initiation factors. The residual EF-G GTPase activity induced by 70 S ribosomes in the presence of saturating amounts of initiation factors/EF-G GTPase inhibitors is higher than EF-G GTPase activity which has been measured when 70 S ribosomes have been substituted by the same amounts of 50 S ribosomal subunits (Table I). Inhibition of EF-G GTPase by IF1 and/or IF3 is more pronounced when 70 S ribosomes are preincubated in the presence of IF1 and/or IF3 prior to the addition of EF-G than without preincubation (Table I). Furthermore, inhibition of 70 S ribosome-induced EF-G GTPase by IF1 and/or IF3 can be overcome by addition of 30 S ribosomal subunits (Table I).

It has been shown that initiation factors IF1 and IF3 cause a dissociation of ribosomal subunits (Subramanian et al., 1969; Sabol et al., 1970; Subramanian and Davies, 1970; Dubnoff and Maiz, 1971; Miall and Tamaoki, 1972; Noll and Noll, 1972). Since the IF1- and IF3-induced dissociation of ribosomal subunits has been reported to be more pronounced at low than at high concentrations of divalent cations, we have investigated the inhibition of EF-G GTPase activity by IF1 and/or IF3 at different Mg2+ concentrations. The inhibition of EF-G GTPase by IF3 but not the effect of IF1 can be reversed by increasing Mg2+ concentrations (Fig. 4).

Modulation of EF-G GTPase Activity by Binding of tRNA, Aminoacyl-tRNA, and Peptidyl-tRNA—The findings that initiation factors IF1 and IF3 inhibit uncoupled EF-G GTPase have induced us to investigate the modulation of EF-G GTPase activity by initiation factor-dependent and non-enzymatic binding of fMet-tRNA^{fMet} and N-acetyl-Phe-tRNA^{fMet}, respectively, to the ribosome. Since both the initiation factor-dependent and the non-enzymatic binding of fMet-tRNA^{fMet} and N-acetyl-Phe-tRNA^{fMet}, respectively, to the ribosome has been reported to be strongly dependent on the ribosomal state (Subramanian et al., 1969; Subramanian and Davies, 1970; Dubnoff and Maiz, 1971; Miall and Tamaoki, 1972; Noll and Noll, 1972).

TABLE I

| Reaction mixtures for preincubation of ribosomes contained in a total volume of 50 μl: 3.0 μmol of Tris-HCl (pH 7.8), 4 μmol of NH4Cl, 0.3 μmol of MgCl2, 0.2 μmol of diethiothreitol, 10 pmol of 70 S ribosomes, and (where indicated) 2 μg of IF1, 1 μg of IF3, 10 pmol of 50 S ribosomal subunits and 10 pmol of 30 S ribosomal subunits. After 10 min at 30 °C, the reaction mixtures were preincubated at 37 °C for 10 min and then incubated with 30 S ribosomal subunits and 10 pmol of [γ-32P]GTP (specific activity 150 cpm/pmol). The amounts of GTP hydrolyzed were determined as described by Sander et al. (1975) and given in nmoles P, released/10 min. |
|----------------|----------------|----------------|----------------|
| First additions | Second additions | Minus preincubation | Plus preincubation |
| IF1 | IF3 | IF1 | IF3 | IF1 | IF3 |
| 50 S | 3.74 | 7.28 | 5.67 | 5.67 |
| 30 S | 6.66 | 9.35 | 5.71 | 5.71 |
| IF1 | 5.52 | 3.45 | 5.52 | 5.52 |
| IF1 | 5.98 | 3.89 | 5.98 | 5.98 |
| IF1 | 6.45 | 5.83 | 6.45 | 6.45 |
| IF3 | 5.97 | 5.82 | 5.97 | 5.97 |
| IF3 | 5.58 | 4.14 | 5.58 | 5.58 |
| IF3 | 5.93 | 4.52 | 5.93 | 5.93 |
| IF3 | 7.52 | 6.96 | 7.52 | 7.52 |
| IF1 + IF3 | 4.83 | 4.02 | 4.83 | 4.83 |
| IF1 + IF3 | 2.97 | 2.69 | 2.97 | 2.97 |
| IF1 + IF3 | 3.61 | 3.39 | 3.61 | 3.61 |
| IF1 + IF3 | 6.34 | 6.01 | 6.34 | 6.34 |
the concentration of divalent cations, the effects on EF-G GTPase activity have been studied at different MgCl₂ concentrations (Fig. 5). All the experiments have been performed in the presence of poly(A, U, G) (Fig. 5, A and C) and poly(U) (Fig. 5, B and D), respectively.

As already reported in the literature (Chiniali and Parmegiani, 1982), addition of fMet-tRNA^Met and N-acetyl-Phe-tRNA^Phe, respectively, results in a stimulation of EF-G GTPase activity at low Mg²⁺ concentration and in an inhibition at high Mg²⁺ concentrations in the absence of initiation factors. Stimulation of EF-G GTPase by binding of peptidyl-tRNA at low Mg²⁺ concentrations is almost reversed by addition of initiation factors IF1 and IF3 (Fig. 5, A and B). The inhibitory effects of initiation factors and peptidyl-tRNA on EF-G GTPase at high Mg²⁺ concentrations, however, have been found to be additive (Fig. 5, A and B). An effect of initiation factor IF2 is observed only at low Mg²⁺ concentrations in the presence of IF1 and IF3 and either fMet-tRNA^Met (in the presence of poly(A, U, G); Fig. 5C) or N-acetyl-Phe-tRNA^Phe (in the presence of poly(U); Fig. 5D). Under these conditions, extremely low EF-G GTPase activities have been measured. These findings clearly demonstrate that EF-G GTPase activity is differentially affected by non-enzymatic and initiation factor-dependent binding of “peptidyl-tRNA,” respectively, to the ribosomal P-site. At low MgCl₂ concentrations, non-enzymatic binding of both aminoacyl-tRNA and peptidyl-tRNA takes place preferentially to the ribosomal P-site (Lucas-Lenard and Lipmann, 1967; Kaji et al., 1989). Under these conditions, EF-G GTPase activity is stimulated (Fig. 5, A and B). In the presence of all the three initiation factors, however, addition of either fMet-tRNA^Met plus poly(A, U, G) or N-acetyl-Phe-tRNA^Phe plus poly(U) results in a very strong inhibition of EF-G GTPase (Fig. 5, C and D). At 6 mM MgCl₂, an initiation factor- and poly(U)-dependent inhibition of EF-G GTPase has been observed for N-acetyl-Phe-tRNA^Phe, but not for Phe-tRNA^Phe or uncharged tRNA^Phe (Table II). In the absence of initiation factors, EF-G GTPase activity is stimulated at 6 mM MgCl₂ not only by addition of N-acetyl-Phe-tRNA^Phe but also by addition of Phe-tRNA^Phe or uncharged tRNA (Table II). At 20 mM MgCl₂, EF-G GTPase activity is inhibited by the poly(U)-dependent binding of N-acetyl-Phe-tRNA^Phe, Phe-tRNA^Phe, or uncharged tRNA both in the absence and presence of initiation factors (Table II).

Inhibition of EF-G GTPase by peptidyl-tRNA, aminoacyl-tRNA, and uncharged tRNA at high MgCl₂ is well known and has been attributed to the binding of tRNA to the ribosomal A-site (Modolell and Vazquez, 1973; Chiniali and Parmeggiani, 1982). It is, therefore, not unexpected that this effect is almost unmodulated by the addition of initiation factors. Considerably more interesting are the differential effects of the mRNA-dependent binding of peptidyl-tRNA to the ribosomal P-site observed at 6 mM MgCl₂ in the presence and absence of initiation factors (Fig. 5; Table II). Therefore, these effects

**Fig. 4.** Effect of MgCl₂ concentration on the ribosome-dependent EF-G GTPase activity in the absence or presence of initiation factors IF1 and/or IF3. 10 pmol of 70 S ribosomes were preincubated at 30 °C in a total volume of 50 μl of ribosome standard buffer in the absence or presence of initiation factors IF1 (6 μg) and/or IF3 (4 μg) and subsequently analyzed for their ability to induce EF-G GTPase, as described under “Experimental Procedures.” ■ control; △, IF1; ○, IF3; ▼, IF1 plus IF3.

**Fig. 5.** Effects of MgCl₂ concentration on the modulation of EF-G GTPase activity by initiation factors and poly(A, U, G)-dependent binding of fMet-tRNA^Met (A and C) or poly(U)-dependent binding of N-acetyl-Phe-tRNA^Phe to the ribosome (B and D). 10 pmol of 70 S ribosomes were preincubated in a total volume of 50 μl of ribosome standard buffer 5 (see “Experimental Procedures”) in the presence of 2.5 μg of poly(A, U, G) (A and C) and 2.5 μg of poly(U) (B and D), respectively, and, where indicated, 4 μg of IF1, 2 μg of IF2, 2 μg of IF3, and/or 50 pmol of fMet-tRNA^Met (A and C) and 80 pmol of N-acetyl-Phe-tRNA^Phe (C and D), respectively. Subsequently, EF-G GTPase activity was measured after addition of 30 pmol of EF-G and 10 nmol of [γ-32P]GTP as described under “Experimental Procedures.” ○, control; △, Δ, plus fMet-tRNA^Met (A and C) or N-acetyl-Phe-tRNA^Phe (B and D); □, ▼, plus IF1, IF3, and fMet-tRNA^Met (A and C) and N-acetyl-Phe-tRNA^Phe (B and D), respectively. The experiments shown in the upper part of the figure (A and B) were performed in the absence of IF2, the experiments shown in the lower part of the figure (C and D) were performed in the presence of IF2.

**Table II**

| Additions | minus initiation factors | plus initiation factors |
|-----------|--------------------------|------------------------|
| 6 mM MgCl₂, 20 mM MgCl₂, 6 mM MgCl₂, 20 mM MgCl₂ | 970 | 600 |
| polynucleotides | 930 | 1,280 | 320 | 710 |
| fMet-tRNA^Met + poly(U) | 2,430 | 1,430 | 290 | 600 |
| Phe-tRNA^Phe + poly(U) | 2,380 | 890 | 1,070 | 780 |
| N-acetyl-Phe-tRNA^Phe + poly(U) | 2,510 | 880 | 460 | 740 |
have been studied at increasing EF-G concentrations (Fig. 6). Stimulation of EF-G GTPase activity by non-enzymatic, poly(U)-dependent binding of N-acetyl-Phe-tRNA\textsuperscript{Phe} is more pronounced at low than at high EF-G concentrations (Fig. 6A). Double-reciprocal plots of these data (Fig. 6B) reveal that stimulation of EF-G GTPase activity by non-enzymatic binding of peptidyl-tRNA to the ribosomal P-site is not accompanied by an increase of $V_{\text{max}}$, while the amount of EF-G needed for $V_{\text{max}/2}$ is reduced. The inhibition of EF-G GTPase activity by initiation factors in the presence or absence of N-acetyl-Phe-tRNA\textsuperscript{Phe} (Fig. 6A), however, results in a reduction of $V_{\text{max}}$ which is more pronounced in the presence than in the absence of N-acetyl-Phe-tRNA\textsuperscript{Phe} (Fig. 6B). Furthermore, the amount of EF-G required for $V_{\text{max}/2}$ is reduced by the addition of initiation factors (Fig. 6B).

The Abilities of Different Ribosome/mRNA/tRNA Complexes to Induce EF-G GTPase—The ribosome-dependent EF-G GTPase activity measured in the presence of synthetic mRNA, peptidyl-tRNA, and/or initiation factors is induced by a mixture of modified and unmodified ribosomes. To eliminate the effects of unmodified 70 S ribosomes and free 50 S ribosomal subunits, we have isolated the different ribosome/mRNA/peptidyl-tRNA complexes (Fig. 7) and compared their abilities to induce EF-G GTPase activity at three different EF-G concentrations. All the different "physiological" ribosome/mRNA/peptidyl-tRNA complexes (Fig. 7) have revealed lower activities than unmodified 70 S ribosomes with respect to the induction of EF-G GTPase, at least at high ratios of [EF-G]/[ribosome] (Table III). The most striking differences between the isolated complexes and unmodified 70 S ribosomes have been observed at low EF-G concentrations (Table III). Again, the most active ribosome species with respect to the induction of EF-G GTPase is the unphysiological complex I\textsuperscript{I} formed by mRNA-dependent, non-enzymatic binding of peptidyl-tRNA, aminoacyl-tRNA, or uncharged tRNA to the ribosomal P-site at low Mg\textsuperscript{2+} concentration (Table III), followed by the artificial complex IV\textsuperscript{V} and the "physiological" complexes III and IV (Fig. 7). Complex III is the natural substrate for EF-G, the "pretranslocational" state of the ribosome formed by the action of the peptidyl transferase. As recently reported by Moazed and Noller (1989), this pretranslocational state is indeed a "hybrid state" of binding, because after the peptidyl transfer, the acceptor ends of the tRNAs have already moved spontaneously from the P- to the E-site and from the P-site to the A-site of the 50 S subunits, respectively. EF-G GTPase only promotes the second step of translocation, the movement of the anticodon ends of the tRNAs relative to the small ribosomal subunit, along with the mRNA (Moazed and Noller, 1989). The resulting complex IV (Fig. 7), is less active than complex III with respect to the induction of EF-G GTPase at low EF-G concentration (Table III). This difference, which is expected on the basis of the model of the ribosomal elongation cycle (Fig. 7), disappears, however, with increasing EF-G concentration. The artificial complex IV\textsuperscript{V}, prepared by treatment of the isolated complex IV with puromycin, reveals a more pronounced induction of EF-G GTPase than complexes III and IV and unmodified 70 S ribosomes, especially when studied at low EF-G concentration.

![FIG. 6. Effect of increasing concentration of EF-G on its ribosome-dependent GTPase activity at 6 mM MgCl\textsubscript{2} in the absence (x) or presence of N-acetyl-Phe-tRNA\textsuperscript{Phe} (.), initiation factors IF1, IF2, and IF3 (a) or both N-acetyl-Phe-tRNA\textsuperscript{Phe} and all the three initiation factors (v). The conditions were the same as in Fig. 5.](image)

![FIG. 7. Ribosomal states during the elongation cycle.](image)

| Table III | Abilities of different ribosome/mRNA/tRNA complexes to induce EF-G GTPase |
|-----------|---------------------------------------------------------------------------|
| Ribosome  | [EF-G]/[ribosome] ratio |
| 50 S subunit | 50 | 6 | 28 | 110 |
| 70 S (untreated) | 70 | 45 | 230 | 380 |
| 70 S + poly(U) | 70 | 48 | 245 | 390 |
| Complex I | 11 | 45 | 47 |
| Complex I\textsuperscript{I} | 107 | 532 | 568 |
| Complex I\textsuperscript{II} | 8 | 36 | 39 |
| Complex I\textsuperscript{III} | 7 | 33 | 35 |
| Complex III | 76 | 368 | 424 |
| Complex IV | 64 | 305 | 402 |
| Complex IV\textsuperscript{V} | 81 | 385 | 478 |
Complex IV
Complex I Low affinity to EF-G. "Inactivation" by EF-Tu-
50 S Ribosomal state
- Translational transition of these complexes to the state of complex III (Fig. 7).
- After EF-T-dependent binding of aminoacyl-tRNA to the ribosomal A-site (complex II) the peptide transfer occurs spontaneously (resulting in complex III), it is not possible to isolate complex II. Therefore, the analogous complexes II* and II** (Fig. 7) are prepared by non-enzymatic binding of N-acetyl-Phe-tRNA^Fm (complex II*) and uncharged tRNA^Fm (complex II**) to complex I at 20 mM MgCl₂.

**DISCUSSION**

The inhibitors of uncoupled EF-G GTPase activity found in the ribosome wash of E. coli (Kurki and Kanno, 1972; Kuriki and Yoshimura, 1974; Voigt and Nagel, 1990) are obviously identical with the initiation factors IF1 and IF3 as recently postulated (Nagel and Voigt, 1992). Our efforts to separate the EF-G GTPase inhibitor activities from these initiation factors have revealed that EF-G GTPase inhibitor activities are perfectly copurified with IF1 and IF3, respectively, under all chromatographic conditions tested (this paper).

The initiation factors IF1 and IF3 have been reported to affect the association-dissociation equilibrium of the ribosomal subunits favouring the dissociation (Subramanian et al., 1968; Sabol et al., 1970; Subramanian and Davies, 1970; Dubnoff and Maitra, 1971; Miall and Tamaoki, 1972; Noll and Noll, 1972; Naakgeboren et al., 1977). Dissociation of ribosomal subunits by initiation factors can be overcome by high Mg²⁺ concentrations (Subramanian et al., 1968). Inhibition of EF-G GTPase activity by IF3, but not the effect of IF1 can be reversed by increasing Mg²⁺ concentrations (Fig. 4). As shown by Naakgeboren et al. (1977), IF1 acts by increasing the rate of exchange of the ribosomal subunits in the 70 S ribosome without changing the position of the equilibrium. Therefore, our finding that the effect of IF1 on EF-G GTPase activity can be reversed by high MgCl₂ concentrations is in accordance with the literature.

The biological significance of the inhibition of EF-G GTPase by initiation factors IF1 and IF3 is obvious; in this way, uncoupled EF-G GTPase activity by 70 S ribosomes, which otherwise should take place also in vivo is presumably abolished (Table IV). A second mechanism to reduce uncoupled EF-G GTPase activity in vivo is, of course, the formation of ribosome/mRNA/tRNA complexes, i.e. the mRNA- and initiation factor-dependent binding of fMet-tRNA^Fm to the ribosomal P-site (complex I; Fig. 7; Table III) and the mRNA-
and EF-Tu-dependent binding of aminoacyl-tRNA to the ribosomal A-site (complex II; Fig. 7; Table III). The relatively high activities observed for complex IV, which is the "product" of EF-G and was therefore expected to have a rather low ability to stimulate EF-G GTPase, is presumably not a problem in vivo. 1) At low EF-G concentrations, complex IV has a considerably lower ability to induce EF-G GTPase than complex III, which is the "substrate" of EF-G (Fig. 7; Table III). 2) Under in vivo conditions, there is a competition between EF-G and EF-Tu for the binding to complex IV.

Therefore, a stoichiometry of 2 molecules of GTP hydrolyzed per peptide bond formed (Cabrer et al., 1976; Chinali and Parmeggiani, 1980; Richter Dahlfors and Kurland, 1990; Voigt and Nagel, 1990) seems to be likely also for in vivo conditions.

With respect to the mechanisms by which EF-G GTPase activity is regulated, the stimulation of EF-G GTPase activity by the non-enzymatic, but mRNA-dependent binding of peptidyl-tRNA, aminoacyl-tRNA, or uncharged tRNA to the ribosomal P-site (Chinali and Parmeggiani, 1982; this paper) is of special interest, although this effect is, of course, not physiological. Since this complex I* has a considerably higher affinity for EF-G than unmodified 70 S ribosomes or ribosome/mRNA complexes (Chinali and Parmeggiani, 1982; this paper), it seems to simulate the natural substrate of EF-G (complex III; Fig. 7). Since the ability of the initiation complex (complex I; Fig. 7) to induce EF-G GTPase is rather low, whereas the activity of complex IV, which is the natural product, is astonishingly high (Fig. 7; Table III), the E-site bound tRNA might have some effect on EF-G GTPase. One possible explanation for the relatively high ability of complex IV to induce EF-G GTPase activity might be a spontaneous back-transition to complex III (Fig. 7), which in vivo is of course abolished by the EF-Tu-dependent binding of aminoacyl-tRNA to the ribosomal A-site. This is, however, no explanation for the high activity of complex I*. It seems to be reasonable to suppose that complex I* is a hybrid state (Moazed and Noller, 1989), which might be more stable than complex III. Since a peptidyl transferase-catalyzed transfer of aminoacyl- and peptidyl-residues to puromycin takes place when aminoacyl-tRNA and peptidyl-tRNA, respectively, are non-enzymatically bound to the ribosomal P-site, we assume that complex I* contains 1 tRNA molecule which is P-site bound and an S subunit and a P-site bound to the 30 S subunit. This complex I* strongly stimulates EF-G GTPase activity but is obviously not converted to a complex I state by the action of EF-G. This model implies that the E-site/P-site bound uncharged tRNA is required for the EF-G-dependent translocation of the ribosome along the mRNA chain.

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**Table IV**

| Ribosomal state | Mechanisms by which uncoupled EF-G GTPase activity is reduced |
|-----------------|---------------------------------------------------------------|
| 50 S subunit    | 50 S subunit induced EF-G GTPase activity is very low at physiological EF-G concentrations. |
| 70 S ribosome   | 70 S ribosomes able to induce EF-G GTPase are almost not present in vivo due to "inactivation" of 30 S subunits by IF1 and IF3 at low concentrations of divalent cations. |
| Complex I       | Low affinity to EF-G. "Inactivation" by EF-Tu-dependent binding of aminoacyl-tRNA. |
| Complex IV      | EF-G binding site "protected." |
| Complex III     | Natural "substrate" for EF-G GTPase. |
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