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Inhibition of Polypeptide Chain Initiation in *Escherichia coli* by Elongation Factor G

(initiation complex/translocase activity)

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ABSTRACT **We have previously reported the isolation from *E. coli* of a specific inhibitor of polypeptide chain initiation that is rendered ineffective when active aminoacylation of transfer RNA is taking place; this is normally the case during natural messenger RNA translation. Surprisingly, the inhibitory activity appears to be a hitherto unrecognized property of the chain elongation factor G. The following hold for preparations purified for either translocase or inhibitor activity: (*1*) equal electrophoretic mobility on polyacrylamide gels; (*2*) equal specific activities for (*a*) inhibition of initiation, (*b*) translocation, and (*c*) ribosome-dependent, uncoupled GTPase; and (*3*) similar heat sensitivity of translocase and inhibitor activities in a temperature-sensitive *E. coli* mutant with an altered elongation factor G. Different sites are apparently involved in translocation and inhibition because the former, but not the latter, is sensitive to *p*-chloromercuribenzoate and fusidic acid.**

Recently we isolated from *Escherichia coli* cells a new inhibitor of polypeptide chain initiation (2). This inhibitor depresses the formation of a 30S or 70S initiation complex as assayed by either AUG- or natural mRNA-directed ribosomal binding of fMet-tRNA$_f$, or poly(U)-directed binding of ac-Phe-tRNA. Chain elongation, e.g., poly(U) translation at high Mg^{2+} concentration, is not inhibited. The inhibitor can apparently be turned on and off, for it is rendered ineffective when active aminoacylation of tRNAs is taking place, e.g., during translation of natural mRNA (MS2 RNA, late T4 RNA). The new inhibitor is distinct from the interference (*i*) factors (3, 4), which have no effect on AUG- or poly(U)-directed initiation but inhibit translation of natural mRNA (5). The results reported in this paper strongly suggest that the inhibitory activity is an intrinsic property of the chain elongation factor G (EF-G).

MATERIALS AND METHODS

Preparations. Virtually homogeneous initiation inhibitor was prepared from *E. coli* MRE 600 essentially as described (2). Two further steps were carried out, chromatography on DEAE–Sephadex A-50 (6) and repeated Agarose filtration. The preparation used throughout this work was derived from the 1.0 M NH_4Cl ribosomal wash. As reported (2), the activity is also present in the high-speed supernatant. Preparations have been made both from this supernatant and the supernatant recovered after centrifugation at 30,000 × *g*. For comparative purposes, EF-G was purified, from *E. coli* MRE 600, by three published procedures (6–8). Homogeneous EF-G, kindly supplied by Dr. J. W. Bodley, University of Minnesota, was used in all experiments of Figs. 1–4. Highly purified preparations of EF-Tu, EF-Ts, and EF-G were generously provided by Dr. H. Weissbach, Roche Institute of Molecular Biology. Homogeneous initiation factors IF-1, IF-2, and unresolved IF-3, as well as unfractionated *E. coli* MRE 600 ribosomes washed with 1.0 M NH_4Cl, were prepared as described (2). All pH values are given at 25°. A temperature-sensitive mutant of *E. coli* with an altered EF-G (9, 10) was kindly furnished by Dr. D. Schlessinger, Washington University (11). This mutant does not grow at 37°; it grows at 30° after a long initial lag. f[^{14}C]Met-tRNA (specific radioactivity, 410 cpm/pmol) and [^{14}C]Phe-tRNA (specific radioactivity, 900 cpm/pmol) were prepared from crude *E. coli* W tRNA. Other preparations and materials were as in previous work (12).

Assays. (*a*) Initiation inhibitor activity was routinely assayed by the AUG-dependent ribosomal binding of f[^{14}C]Met-tRNA$_f$ as the test system (2). The assay was conducted exactly as described earlier (ref. 2, legend to Fig. 1). (*b*) Translocase activity was assayed as the poly(U)-directed synthesis of [^{14}C]-polyphenylalanine from [^{14}C]Phe-tRNA, at high (6.6 mM) Mg^{2+} concentration. In the presence of excess EF-Tu and EF-Ts, the reaction rate is proportional to the concentration of EF-G within a narrow concentration range; no initiation factors are required. The assay was carried out exactly as described earlier (ref. 2, legend to Table 3, "nonenzymatic" assays) with 2.5 μg of EF-Tu, 3 μg of EF-Ts, and variable amounts of either authentic EF-G or protein purified for initiation inhibitor activity. (*c*) Ribosome-dependent, uncoupled GTPase was assayed with [γ-^{32}P]GTP as described by Kaziro *et al.* (6). Radioactivity was measured in Liquifluor in a Packard Tri-Carb scintillation spectrometer.

Miscellaneous. Polyacrylamide gel electrophoresis was conducted both under nondissociating conditions (13) and in sodium dodecyl sulfate ($DodSO_4^-$) (14). In the former case Tris-glycine, pH 9.6, was the top buffer and Tris·HCl, pH 8.9, the bottom buffer; the runs were conducted at 5 mA per gel, with 7.5% gels. $DodSO_4^-$ gel electrophoresis was carried

Abbreviations: The designation of prokaryotic initiation factors as IF-1, IF-2, and IF-3, and of elongation factors as EF-Tu, EF-Ts, and EF-G conforms with currently accepted nomenclature (1).

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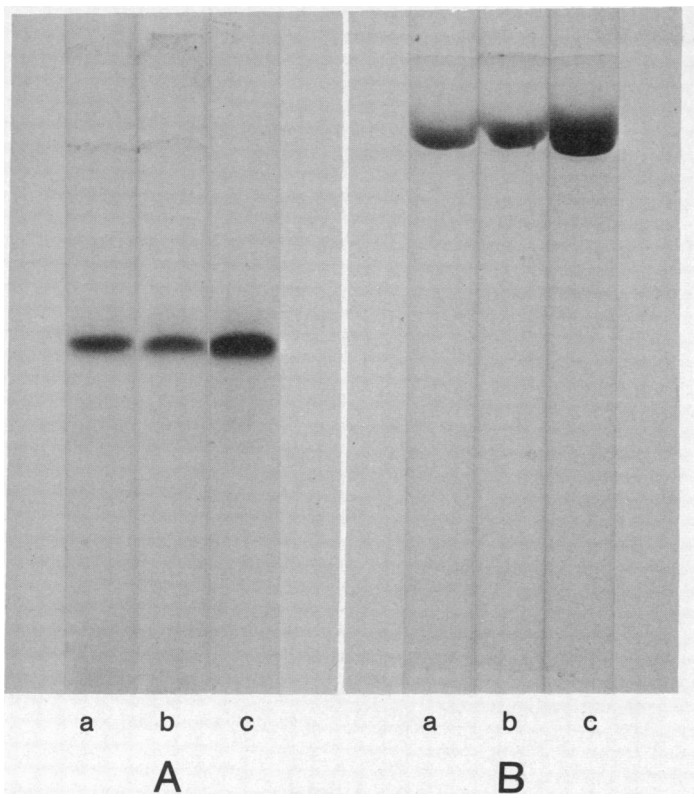

FIG. 1. Polyacrylamide gel electrophoresis of initiation inhibitor and authentic EF-G. (*A*) Nondissociating conditions, pH 9.3; (*B*) in $DodSO_4^-$, pH 7.0. (*a*) Preparation purified for initiation inhibitor activity (10 μg); (*b*) authentic EF-G (10 μg); (*c*) coelectrophoresis of *a* and *b* (each 10 μg).

out in 0.1 M phosphate buffer, pH 7.0; the concentration of $DodSO_4^-$ was 0.1%. The runs were done at 8 mA per gel, with 10% acrylamide, 0.27% methylene-bisacrylamide gels. The gels were stained with 0.25% Coomassie brilliant blue in 45.4% methanol–9.2% acetic acid and destained in 7.5% acetic acid–5% methanol at 70°.

Molecular weights were determined by $DodSO_4^-$–gel electrophoresis (14) and by gel filtration on Sephadex G-200 (15). The markers used in the former case were *E. coli* β-galactosidase (P-L Biochemicals, 4 subunits, each 130,000 daltons), phosphorylase (Sigma, 4 subunits, each 94,000 daltons), bovine-serum albumin (68,000 daltons), immunoglobulin H chain (50,000 daltons), ovalbumin (43,000 daltons), chymotrypsinogen (25,700 daltons), immunoglobulin L chain (23,500 daltons), and myoglobin (17,800 daltons). The markers used in gel filtration were *E. coli* RNA polymerase‡ (880,000 daltons), immunoglobulin (160,000 daltons), serum albumin, ovalbumin and chymotrypsinogen. Unless otherwise stated, the marker proteins were purchased from Schwarz/Mann Biochemicals. Protein was determined by the Lowry procedue (16).

RESULTS

Preparations purified for initiation inhibitor activity were compared with authentic EF-G as regards physical and biological properties.

‡ S. Lee-Huang and H. Lee, unpublished procedure.

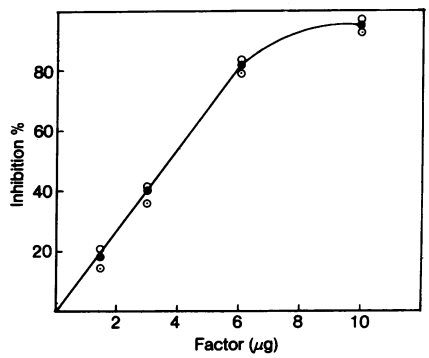

FIG. 2. Inhibition of AUG-dependent ribosomal binding of f[^{14}C]Met-tRNA as a function of the concentration of inhibitor or authentic EF-G. (○) protein purified for initiation inhibitor activity; (●) EF-G (Bodley); (⊙) EF-G (Weissbach).

Physical Properties. As seen in Fig. 1, the electrophoretic mobilities of the initiation inhibitor and authentic EF-G were the same under nondissociating and dissociating conditions. Both were virtually homogeneous by this criterion. Their molecular weights were also identical. Each protein gave a molecular weight of 110,000 by gel filtration and 83,000 by $DodSO_4^-$–gel electrophoresis. The higher value by gel filtration might be due to partial aggregation. Values of 83,000 (6) and 84,000 daltons (7), as determined by equilibrium sedimentation under nondissociating conditions, have been reported for EF-G (see also refs. 6 and 17).

Biological Properties. As seen in Fig. 2, authentic EF-G and preparations purified for inhibition of the AUG-dependent ribosomal binding of fMet-tRNA had identical inhibitory activity on this reaction. Fig. 3 shows that the same preparations had identical translocase activity, as assayed by poly(U) translation. Moreover, as shown in Fig. 4, inhibitor and authentic EF-G had identical ribosome-dependent GTPase activity.

Experiments with Temperature-Sensitive Mutant Having an Altered EF-G. Protein fractions from *E. coli* MRE 600 (wild type) and the temperature-sensitive mutant, purified simultaneously from the high-speed supernatant (105,000 × *g*) for

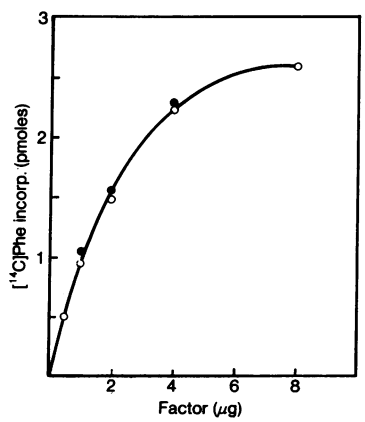

FIG. 3. Translocase activity [poly(U) translation] as a function of the concentration of inhibitor or authentic EF-G. (○) Protein purified for initiation inhibitor activity; (●) EF-G (Bodley). A blank value of 0.12 pmol of [^{14}C]phenylalanine incorporated in the absence of inhibitor or EF-G was subtracted throughout. The corresponding value with 8 μg of EF-G, but without EF-Tu and EF-Ts, was 0.07 pmol.

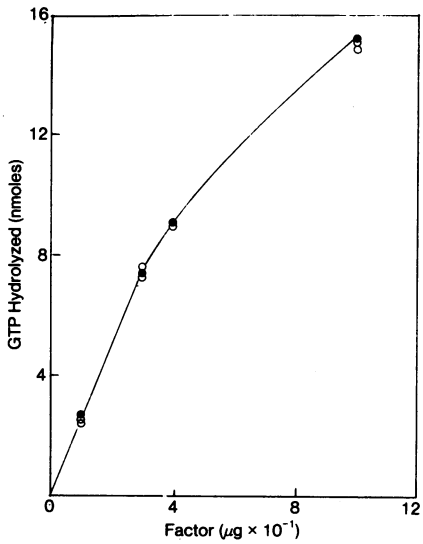

FIG. 4. Ribosome-dependent, uncoupled GTPase activity as a function of the concentration of inhibitor or authentic EF-G. (○) Protein purified for initiation inhibitor activity; (●) EF-G (Bodley); (⊙) EF-G (Weissbach).

initiation inhibitor activity through the DEAE–cellulose step (2), were assayed for translocase and inhibitor before and after heating for 5 min at 54°. As seen in Table 1, both activities were markedly heat-sensitive in the mutant, although inhibitor was significantly less sensitive than translocase. The mutant EF-G appeared to be readily denatured in an irreversible fashion, and could not be purified beyond the DEAE–cellulose step without considerable loss of activity. Contrary to wild type, the translocase and inhibitor activities of the mutant preparation decline sharply upon storage in 50% glycerol at −20°.

Effect of p-Chloromercuribenzoate and Fusidic Acid. p-Chloromercuribenzoate, even at much higher concentrations

TABLE 1. *Translocase and inhibitor activity of preparations from wild-type and temperature-sensitive E. coli mutant*

Source of EF-G	Heating	Translocase: [^{14}C]-Phe incorporation (pmol)	Translocase: Loss on heating (%)	Inhibitor activity: f[^{14}C]-Met-tRNA binding (pmol)	Inhibitor activity: Inhibition (%)	Inhibitor activity: Loss on heating (%)
None				9.6*		
Wild type	No	6.2		0	100	
Wild type	Yes	4.9	21	0.7	93	7
Mutant	No	3.0		4.8	50	
Mutant	Yes	0.2	93	7.7	20	60

Poly(U) translation (EF-G activity) and AUG-dependent ribosomal binding of f[^{14}C]Met-tRNA (inhibitor activity) were assayed as described in *Materials and Methods*. The EF-G preparations used (each 100 μg of protein per sample) were DEAE–cellulose fractions. For heating, samples containing 14 mg of protein per ml were kept at 54° for 5 min and chilled in ice. Any precipitate formed was removed by centrifugation at 4°, and the supernatants were adjusted to a protein concentration of 10 mg/ml.

* Control value in the absence of inhibitor (EF-G).

TABLE 2. *Differential effect of p-chloromercuribenzoate (ClHgBz) on translocase and inhibitor activities of EF-G*

EF-G (μg)	ClHgBz (mM)	Translocase: [^{14}C]Phe incorporation (pmol)	Translocase: Change due to ClHgBz (%)	Inhibitor activity: f[^{14}C]-Met-tRNA binding (pmol)	Inhibitor activity: Inhibition (%)	Inhibitor activity: Change due to ClHgBz (%)
0	0	0.02		6.65		
4.0	0	2.36		1.39	79	
4.0	0.2	0.30	−87	0.65	90	+14
4.0	1.4			0.65	90	+14

* Assays as in Table 1 with factor (EF-G) purified for inhibitor activity.

than needed to produce considerable inhibition of the translocase, had no significant effect on the inhibitor activity (Table 2). The initiation inhibitor activity in factor preparations from wild-type *E. coli* was insensitive to even high concentrations of fusidic acid, but preparations from the mutant were somewhat less resistant to the antibiotic (Table 3). It may be noted (Table 3) that the fusidic acid sensitivity of translocase was much higher in the mutant than in the wild-type preparation.

DISCUSSION

The results presented in this paper strongly suggest that the activity responsible for inhibition of polypeptide chain initiation in *E. coli* systems (2) resides in the chain elongation factor G. The initiation inhibitor, it may be recalled, can apparently be turned on and off and is, in fact, rendered ineffective when active aminoacylation of tRNA is taking place, as during translation of natural mRNA. Under these conditions, EF-G functions as translocase. This feature makes possible the coexistence of initiation inhibitory and translocation activities in the same molecule. Since formation of the 30S initiation complex is also inhibited (2) the factor might act by binding to 30S subunits. It might also act by complexing with and inactivating IF-2, but this is perhaps made unlikely by the fact that the factor also inhibits, although to a lesser extent, the nonenzymatic, AUG-dependent ribosomal binding of fMet-tRNA at high (20 mM) Mg^{2+} concentration, in the absence of IF-1 and IF-2 (2). In any case, whereas EF-G acts catalytically with respect to ribosomes in translocation, at least an equimolar ratio of factor to ribosomes is required for maximal inhibition. The occurrence of large amounts of EF-G in *E. coli* [about 2.5% of the protein in crude extracts (6)] is compatible with such a function. From Fig. 2 it may be seen that 6 μg of EF-G caused 80% inhibition with 2 A_{260} units (133.4 μg) of ribosomes. For a molecular weight of 83,000 for EF-G and 2.7×10^6 for the 70S ribosome, this corresponds to a molar EF-G to 70S ribosome ratio of 1.4.

A fusidic acid-dependent inhibition of initiation complex formation on *E. coli* ribosomes by EF-G, with crude initiation factors, has been reported (18). However, substantial inhibition without fusidic acid was later observed with purified initiation factors§. This result is in line with our own observations.

§ G. Chinali and A. Parmeggiani, personal communication.

TABLE 3. *Effect of fusidic acid on translocase and inhibitor activity of EF-G from wild-type and mutant E. coli*

Source of EF-G	Fusidic acid (mM)	Translocase: [^{14}C]-Phe incorporation (pmol)	Translocase: Inhibition by fusidic acid (%)	Inhibitor activity: f[^{14}C]-Met-tRNA binding (pmol)	Inhibitor activity: Inhibition (%)	Inhibitor activity: Inhibition by fusidic acid (%)
None				9.4		
Wild type	0	6.3		0	100	
Wild type	1.66	2.8	56	0	100	0
Wild type	0.33	4.0	37			
Wild type	0.166	4.9	22			
Wild type	0.083	5.1	19			
Wild type	3.22			0.3	97	3
Mutant	0	2.9		4.5	52	
Mutant	1.66			4.7	50	4
Mutant	0.33	0.09	97			
Mutant	0.166	0.14	96			
Mutant	0.083	0.33	89			
Mutant	3.22			5.7	39	25

Assays as in Table 1 with similar EF-G preparations.

The mechanism of inhibition is unknown, as is the mechanism of inhibition release connected with aminoacylation of tRNAs. The occurrence of such a release makes it possible to think that EF-G may have a regulatory role in prokaryotic protein synthesis by preventing initiation under conditions of amino acid or tRNA shortage.

The fact that *p*-chloromercuribenzoate and fusidic acid, both strong inhibitors of translocase, do not affect the inhibitory activity indicates that, if the two activities do indeed coexist in the EF-G molecule, different active sites must be involved. It may be remembered in this connection that the inhibitor activity of the temperature-sensitive mutant, although markedly heat-labile, was somewhat less so than the translocase activity.

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