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ARTICLE *in* BIOCHIMICA ET BIOPHYSICA ACTA · FEBRUARY 1992

Impact Factor: 4.66 · DOI: 10.1016/0167-4781(92)90478-I · Source: PubMed

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Biochimica et Biophysica Acta, 1129 (1992) 145–148

BBAEXP 92318

An inhibitor of elongation factor G (EF-G) GTPase present in the ribosome wash of *Escherichia coli*: a complex of initiation factors IF1 and IF3?

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(Received 6 June 1991)

Key words: Elongation factor G; Inhibitor; GTPase (EC 3.6.1.-); (*E. coli*)

An inhibitor of elongation factor G (EF-G) GTPase isolated from the ribosome wash of *Escherichia coli* was shown to stimulate the poly(A,U,G)- and initiation factor 2 (IF2)-dependent binding of *N*-formyl-[^{35}S]Met-tRNA^{fMet} to ribosomes. In the presence of saturating amounts of the EF-G GTPase inhibitor, neither addition of initiation factor 1 (IF1) nor addition of initiation factor 3 (IF3) caused a further stimulation of the formation of *N*-formyl-[^{35}S]Met-tRNA^{fMET}/poly(A,U,G)/ribosome complexes. Both IF1 and IF3 were shown to inhibit ribosome-dependent EF-G GTPase, especially when both initiation factors were added either in absence or in the presence of initiation factor 2 (IF2), poly(A,U,G) and *N*-formyl-Met-tRNA^{fMet}. Therefore, we conclude that the EF-G GTPase inhibitor consisting of two polypeptide subunits with apparent molecular masses of 23000 and 10000 Da is a complex of initiation factors IF1 and IF3. The inhibition of EF-G GTPase by IF3, but not the effects of IF1 in the presence or absence of IF3 could be reversed by increasing the Mg^{2+}-concentration as already shown for the EF-G GTPase inhibitor. Therefore, IF1 as well as the EF-G GTPase inhibitor do not influence the ribosome-dependent EF-G GTPase by affecting the association of ribosomal subunits.

Introduction

The ribosome wash of *Escherichia coli* was shown to contain two inhibitors of the ribosome-dependent GTP hydrolysis by elongation factor (EF) G [1–3]. One of these inhibitors was purified to homogeneity and characterized [3]. The isolated inhibitor was found to consist of two polypeptide subunits with apparent molecular masses of 23000 and 10000 Da. Poly(U)-dependent poly-(phenylalanine) synthesis was found to be considerably less sensitive to the inhibitor than EF-G GTPase, especially when ribosomes were preincubated in the presence of poly(U) and Phe-tRNA^{Phe} [3]. Competition experiments with EF-G, ribosomes and ribosomal subunits indicated that the target site of the inhibitor is the 30S ribosomal subunit [3]. Therefore, we have investigated whether or not this inhibitor of EF-G GTPase interferes with polypeptide chain initiation. As shown in the present communication, this inhibitor stimulated the initiation factor 2 (IF2)-dependent formation of *N*-formyl-[^{35}S]Met-tRNA^{fMet}/poly(A,U,G)/ribosome complexes. At high inhibitor concentrations, the initiation factor 2 (IF2)-dependent formation of *N*-formyl-[^{35}S]Met-tRNA^{fMet}/poly(A,U,G)/ribosome complexes was no longer stimulated by addition of initiation factors IF1 and/or IF3. Furthermore, initiation factors, especially IF1 and IF3, were found to inhibit the uncoupled EF-G GTPase. These findings indicate that this inhibitor of uncoupled EF-G GTPase might be a complex of initiation factors IF1 and IF3.

Materials and Methods

Materials

ATP (Na^+ salt), GTP (Li^+ salt) and a tRNA mixture from *E. coli* MRE 600 were obtained from Boehringer (Mannheim, Germany). GTP was passed through a column of AG 50W-X2 cation exchange resin (H^+-form, Bio-Rad Laboratories, Richmond, CA,

Abbreviations: EF-G, elongation factor G; IF, initiation factor.

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U.S.A.). Folinic acid and poly(A,U,G) were supplied by Sigma (Deisenhofen, Germany). L-[^{35}S]Methionine (specific activity 800 Ci/mmol) and carrier-free $^{32}P_i$ were obtained from New England Nuclear (Dreieich, Germany) and Amersham-Buchler (Braunschweig, Germany). [γ-^{32}P]GTP was prepared by the method of Glynn and Chappel [4,5] and purified by elution from DEAE-cellulose (bicarbonate form) with a gradient of triethylammonium bicarbonate buffer [6].

Ribosomes, elongation factor (EF) G and EF-G GTPase inhibitor were prepared as recently described [3]. Initiation factors IF1, IF2 and IF3 were isolated from the ribosome wash of *E. coli* according to Wahba and Miller [7]. *E. coli* MRE 600 tRNA was charged with L-[^{35}S]methionine (specific radioactivity 100 Ci/mmol) in the presence of ^{5}*N*-formyl-tetrahydrofolic acid (= folinic acid) and a dialysed *E. coli* MRE 600 S150 fraction [8].

Poly(A,U,G)-dependent binding of N-formyl-[^{35}S]Met-tRNA^{fMet} to ribosomes

AUG-dependent binding of *N*-formyl-[^{35}S]Met-tRNA^{fMet} to ribosomes was determined essentially as described by Wahba and Miller [7] with the exception that poly(A,U,G) instead of ApUpG was used as synthetic mRNA.

GTPase assays

GTPase activities were measured as recently described [3]. The reaction mixtures contained in a final volume of 75 μl: 4.5 μmol Tris-HCl (pH 7.8), 1 μmol $MgCl_2$, 6 μmol NH_4Cl, 0.2 μmol dithiothreitol, 10 nmol [γ-^{32}P]GTP (specific activity 150 cpm/pmol), 10 pmol ribosomes and 50 pmol EF-G. The reaction was started by incubation at 30 °C and was stopped after 10 min by chilling in iced water. To each sample 5 μl 50 mM KH_2PO_4 and 75 μl 1 M $HClO_4$ were added. The liberated $^{32}P_i$ was extracted and measured as described elsewhere [9].

Results and Discussion

An inhibitor of the uncoupled GTPase activity of polypeptide chain elongation factor G (EF-G) was recently isolated from the ribosome wash of *E. coli* [3]. The target site of this inhibitor is the 30S ribosomal subunit [3]. Therefore, we have investigated whether or not this inhibitor also affects polypeptide chain initiation.

In the presence of initiation factors IF1, IF2 and IF3, the poly(A,U,G)-dependent binding of *N*-formyl-[^{35}S]Met-tRNA^{fMet} to ribosomes was not inhibited by addition of the Ef-G GTPase inhibitor, but a stimulation of 50% was observed (Table I). In the absence of poly(A,U,G) or IF2, the binding of *N*-formyl-[^{35}S]Met-tRNA^{fMet} to ribosomes was not affected by addition of the EF-G GTPAse inhibitor (Table I). Under the conditions used, binding of *N*-formyl-[^{35}S]Met-tRNA^{fMet} to ribosomes was completely dependent on the presence of both poly(A,U,G) and IF2.

TABLE I

Effect of EF-G GTPase inhibitor on poly(A,U,G)-dependent binding of N-formyl-[^{35}S]Met-tRNA^{fMet} to ribosomes

The complete reaction mixtures contained in a final volume of 50 μl: 5 μmol NH_4Cl, 0.25 μmol $MgCl_2$, 0.1 μmol dithiothreitol, 2.5 μmol Tris-HCl (pH 7.2), 10 nmol GTP, 2 μg poly(A,U,G), 20 pmol *N*-formyl-[^{35}S]Met-tRNA^{fMet} (50–100 Ci/mmol), 2 μg of IF1, 0.2 μg of IF2, 1 μg of IF3 and, where indicated, 5 μg of EF-G GTPase inhibitor. The reaction mixtures were preincubated for 5 min at 25 °C prior to the addition of *N*-formyl-[^{35}S]Met-tRNA^{fMet}. After addition of the aminoacyl-tRNA, the reaction mixtures were incubated at 25 °C for 15 min. The reaction was stopped by the addition of 1 ml of ice-cold wash buffer containing 100 mM NH_4Cl, 5 mM $MgCl_2$ and 50 mM Tris-HCl (pH 7.8). The solutions were then filtered through Millipore filters [7]. The filters were washed three times with 1 ml of wash buffer, dried and the radioactivity measured in a Packard Tri-Carb liquid scintillation spectrometer.

Experimental system	Inhibitor	*N*-formyl-[^{35}S] Met-tRNA bound (pmol)	Inhibitor effect (%)
Complete	–	0.93	
Complete	+	1.41	+51%
Minus poly(A,U,G)	–	0.01	
Minus poly(A,U,G)	+	0.01	±0%
Minus ribosomes	–	< 0.01	
Minus ribosomes	+	< 0.01	±0%
Minus IF2	–	0.04	
Minus IF2	+	0.04	±0%
Minus IF1	–	0.32	
Minus IF1	+	1.15	+260%
Minus IF3	–	0.64	
Minus IF3	+	1.32	+106%

TABLE II

Effects of increasing amounts of EF-G GTPase inhibitor on the IF2-dependent formation of N-formyl-[^{35}S]Met-tRNA-poly(A,U,G)-ribosome complexes

The experimental conditions were the same as in Table I.

Inhibitor (μg protein)	Initiation factors	*N*-formyl-[^{35}S] Met-tRNA bound (pmol)	Stimulation (%)
0	IF2	0.23	(= 100%)
0	IF2 + IF1 + IF3	0.84	265
0.4	IF2	0.31	35
0.4	IF2 + IF1 + IF3	0.94	308
1.1	IF2	0.65	182
1.1	IF2 + IF1 + IF3	1.02	343
2.2	IF2	0.97	321
2.2	IF2 + IF1 + IF3	1.09	373
4.5	IF2	1.15	400
4.5	IF2 + IF1 + IF3	1.12	387
6.7	IF2	1.30	465
6.7	IF2 + IF1 + IF3	1.08	370

In the absence of IF1 or IF3, addition of the EF-G GTPase inhibitor caused a stimulation of the poly(A,U,G)- and IF2-dependent binding of *N*-formyl-[^{35}S]Met-tRNA^{fMet} to ribosomes by 250 and 106%, respectively (Table I). These findings show that the EF-G GTPase inhibitor stimulates the poly(A,U,G)- and IF2-dependent binding of *N*-formyl-[^{35}S]Met-tRNA^{fMet} to ribosomes and that this stimulation is more prominent in the absence of IF1 or IF3 than in the presence of these initiation factors.

A stimulation of poly(A,U,G)- and IF2-dependent binding of *N*-formyl-[^{35}S]Met-tRNA^{fMet} by the EF-G GTPase inhibitor was also observed in the absence of both IF1 and IF3 (Table II). At high concentrations of the EF-G GTPase inhibitor, addition of initiation factors IF1 and IF3 no longer caused a further stimulation of poly(A,U,G)- and IF2-dependent binding of *N*-formyl-[^{35}S]Met-tRNA^{fMet} to ribosomes (Table II). Therefore, the EF-G GTPase inhibitor is able to substitute the effects of IF1 and IF3 on polypeptide chain initiation – at least with synthetic mRNA.

The EF-G GTPase inhibitor was shown to consist of two polypeptide subunits with apparent molecular masses of 23000 and 10000 Da [3]. The initiation factors IF1 and IF3 have been shown to consist of single polypeptide chains with apparent molecular masses of 9000–10000 Da and 21000–23000 Da, respectively [10–12]. Therefore, the inhibitor of EF-G GTPase isolated from the ribosome wash of *E. coli* might be a complex of initiation factors IF1 and IF3. For this reason, we have investigated the effects of initiation factors on the ribosome-dependent GTP hydrolysis by elongation factor (EF) G (Table III).

TABLE III

Effects of initiation factors on the ribosome-dependent GTP hydrolysis by elongation factor (EF) G

The reaction mixtures contained in a final volume of 75 μl: 4.5 μmol Tris-HCl (pH 7.8), 1 μmol $MgCl_2$, 6 μmol NH_4Cl, 0.2 μmol dithiothreitol, 10 nmol [γ-^{32}P]GTP (specific activity 150 cpm/pmol), 10 pmol ribosomes, 50 pmol EF-G and, where indicated, 2 μg poly(A,U,G), 20 pmol *N*-formyl-Met-tRNA^{fMet}, 1 μg of IF2, 2 μg of IF1 and/or 1 μg of IF3. The amounts of GTP hydrolyzed were determined as described elsewhere [3].

Additions	GTP hydrolyzed (nmol)	
	−EF-G	+EF-G
+Ribosomes	0.07	9.55
+IF2−ribosomes	1.14	1.16
+IF2+ribosomes	4.45	8.43
+IF1+ribosomes	0.05	5.49
+IF3+ribosomes	0.08	7.22
+IF1+IF3+ribosomes	0.03	3.97
+IF2+ribosomes+poly(A,U,G) + *N*-formyl-Met-tRNA	2.20	5.9[illegible]
+IF1+IF2+IF3+ribosomes +poly(A,U,G)+ *N*-formyl-Met-tRNA	1.46	2.50

In reaction mixtures containing ribosomes, IF 2 and EF-G, about the same amounts of GTP were hydrolyzed as in the absence of IF2. However, a considerable proportion of this GTP hydrolysis has to be attributed to IF2-dependent GTPase (Table III). Part of this IF2-dependent activity was found to be ribosome independent (Table III). It has to be concluded that ribosome-dependent GTP hydrolysis by elongation factor (EF) G is reduced by the addition of initiation factor 2. This finding is not unexpected as it has been reported that the ribosomal binding sites for IF2 and elongation factors G and Tu share certain common determinants including the L7 and L12 proteins [13–19]. Furthermore, the ribosome-dependent GTPase activities of both EF-G and IF2 are inhibited by thiostrepton [20,21]. Ochoa and coworkers have shown that an inhibitor of polypeptide chain initiation [22] is identical with elongation factor (EF) G [23].

The effects of initiation factors IF3 and especially IF1 on the EF-G GTPase were found to be more prominent than the effects of IF2 (Table III) because IF1 and IF3 have no GTPase activities. Both addition of initiation factors IF1 and IF3 considerably reduced the ribosome-dependent GTP hydrolysis by EF-G (Table III). These findings clearly show that initiation factors IF1 and IF3 are inhibitors of the uncoupled EF-G GTPase.

The conclusions that initiation factors specifically inhibit the uncoupled EF-G GTPase is further corroborated by our finding that, in the presence of poly(A,U,G) and *N*-formyl-[^{35}S]Met-tRNA^{fMet}, addition of the mixture of initiation factors IF1, IF2 and IF3 substantially reduced the EF-G GTPase activity. Therefore, we conclude that the coupling of ribosome-dependent GTP hydrolysis by elongation factor G to polypeptide elongation is due to the formation of 'correct' peptidyl-tRNA/mRNA/ribosome complexes. So far, our findings are in agreement with the observations of Vasquez and coworkers that uncoupled ribosome-dependent EF-G GTPase activity was considerably reduced when ribosomes were substituted by isolated polysomes [24].

Uncoupled EF-G GTPase is considerably stimulated by the association of ribosomal subunits [25–27]. On the other hand, initiation factors IF1 and especially IF3 favour the dissociation of ribosomal subunits at low concentrations of divalent cations [28–30]. Since the initiation factor-induced dissociation of ribosomal subunits can be reversed by increasing Mg^{2+}-concentrations [28–30], we have investigated the effects of different $MgCl_2$-concentrations on the inhibition of uncoupled EF-G GTPase by initiation factors IF1 and IF3. As shown in Table IV, the inhibition of EF-G GTPase by IF3 could be overcome by increasing concentrations of divalent cations, whereas the effects of IF1 in the presence or absence of IF3 could not be

TABLE IV

Effects of Mg^{2+}-concentration on the inhibition of ribosomes-dependent EF-G GTPase by IF1, IF3 and EF-G GTPase inhibitor

The reaction mixture contained in a final volume of 75 μl: 4.5 μmol Tris-HCl (pH 7.8), 6 μmol NH_4Cl, 0.2 μmol dithiothreitol, 10 nmol [γ-^{32}P]GTP (specific activity 150 cpm/pmol), 10 pmol ribosomes, 50 pmol EF-G, $MgCl_2$ as indicated and, if present, 2 μg of IF1, 1 μg of IF3 or 4 μg of EF-G GTPase inhibitor. The amounts of GTP hydrolyzed were determined as described elsewhere [3] and given in nmol GTP hydrolyzed per 10 min.

Additions	$MgCl_2$-concentration			
	5 mM	10 mM	15 mM	20 mM
Control	5.32	10.17	11.84	9.26
+ IF1	2.55	5.64	6.87	6.63
+ IF3	4.91	7.33	9.40	9.12
+ IF1 + IF3	1.62	3.37	4.19	3.91
+ EF-G GTPase inhibitor	1.55	3.14	4.02	3.05

reversed by increasing $MgCl_2$-concentrations as already shown for the EF-G GTPase inhibitor (Table IV; Ref. 3). Therefore, these factors do not primarily influence the uncoupled EF-G GTPase activity by affecting the association–dissociation equilibrium of ribosomal subunits.

Since uncoupled EF-G GTPase is strongly influenced by the conformation of the ribosome as shown by Vasquez and coworkers [24] and the data presented in this communication, a comparative study of different tRNA-mRNA-ribosome complexes [31–33] as 'substrates' for EF-G should provide an insight into the regulation of EF-G GTPase activity as well as into the mechanism of the coupling between EF-G GTPase activity and translocation.

Acknowledgement

The authors wish to thank Dr. Andrea Parmeggiani (present address: Laboratoire de Biochimie, École Polytechnique, Palaiseau, France), in whose laboratory this project was initiated several years ago, for support and laboratory facilities.

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