INHIBITION BY THIOSTREPTON OF THE IF-2-DEPENDENT RIBOSOMAL GTPase*

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1. Introduction

During protein synthesis GTP hydrolysis occurs at the initiation and elongation steps [1]; it has also been shown at the termination step in eukaryotic cells [2]. A GTP hydrolysis not coupled with protein synthesis (in the absence of mRNA and tRNA) can also be observed: whereas in the presence of either ribosome or any factor alone very little GTP hydrolysis can be observed, significant hydrolysis occurs in the presence of ribosomes and one of the factors involved in any one of the steps: initiation factor IF-2, or elongation factor EF-G [3], or termination factor TF-R in eukaryotic cells [4–6]. Some ribosome-dependent GTP hydrolysis also occurs in thermophilic bacteria in the presence of EF-Tu [7].

The significance of these uncoupled GTP hydrolyses is not clear.

Two groups of inhibitors of the factor and ribosome dependent GTP hydrolysis are known: the first, fusidic acid, prevents EF-G-dependent GTP hydrolysis by stabilizing the ternary complex: ribosome–EF-G–GDP (which is formed after one round of EF-G-promoted GTP hydrolysis), thus blocking any further GTP hydrolysis [8, 9]. This antibiotic has no effect on the ribosomal GTPase promoted by IF-2 [5], EF-T [7], or TF-R [2]. The second group includes thiostrepton and siomycin. These antibiotics inhibit EF-G and EF-T-dependent GTP hydrolysis by quite a different mechanism: they irreversibly inactivate the binding site for EF-G, thus preventing the formation of the ribosome–EF-G–GTP (or GDP) complex [10–13]. These antibiotics also prevent binding of aminoacyl-tRNA to the A site [14]. Moreover, siomycin-treated ribosomes cannot exhibit the GTPase activity associated with EF-G, nor that associated with EF-T [14]. These effects seem to result from a single action of siomycin on the 50 S ribosome subunit, suggesting a relationship between EF-G binding site and that of the aminoacyl-tRNA–EF-Tu–GTP complex.

Neither siomycin nor thiostrepton bind to the 30 S subunits, and the reported lack of inhibition of fMet-tRNA binding [14] by thiostrepton supports the view that during initiation this tRNA does not go through the A site on the ribosome [15, 16]. In the present communication we report that, despite its failure to inhibit fMet-tRNA binding, thiostrepton does inhibit the GTPase activity catalyzed by IF-2.

2. Materials and methods

Ribosomes washed with ammonium chloride (M) and purified initiation factors, IF-1 and IF-2 from *E. coli* MRE 600, were prepared as described elsewhere [5].

Elongation factors, EF-T(Tu+Ts) and EF-G were prepared in our laboratory by M. Springer, as described previously [17].

Unfractionated *E. coli* B tRNA (General Biochemicals) was charged with ^{3}H-methionine (specific activity, 1 Ci/mmole) and formylated; or charged with ^{14}C-phenylalanine (specific activity, 235 mCi/mmole) according to the method already described [5]. The labelled amino acids were products of C.E.A., Saclay, France.

* A preliminary report was given in a lecture delivered at a Symposium of the Société de Chimie Biologique [25].

Binding of aminoacyl-tRNA to ribosomes was assayed by filtration on nitrocellulose filters.

GTPase activity was assayed as already described [5].

Thiostrepton was added to saturation in dimethyl sulphoxide (Me_2SO, 20%), consequently between 4 and 5% Me_2SO was present in the reaction mixture with the antibiotic. The effect of Me_2SO will be discussed in the results.

Poly A, U, G, poly U, and ApUpG were synthesized by polynucleotide phosphorylase in the usual manner [18].

Fusidic acid, sodium salt, was a gift from Laboratoire Leo, Paris, and thiostrepton was a gift from Dr. Modolell; we are grateful to both.

3. Results

3.1. *Lack of effect of thiostrepton on initiation*

Like siomycin, thiostrepton, in solution in Me_2SO, does not inhibit the binding of ^{3}H-fMet-tRNA to 70 S washed ribosomes stimulated by IF-1 + IF-2, even in 5 to 10-fold excess (in molar equivalents). The fMet-tRNA bound in the presence of thiostrepton reacts with puromycin, and is released by streptomycin; this indicates that the antibiotic does not interfere with fMet-tRNA binding at P site (table 1). By contrast, and confirming previous reports, Phe-tRNA binding stimulated by EF-T is inhibited by thiostrepton (data not shown). In the absence of the antibiotic, Me_2SO alone stimulates the binding of fMet-tRNA and of Phe-tRNA to about the same extent.

Table 1
Puromycin reaction and streptomycin action on fMet-tRNA bound to ribosomes in the presence of thiostrepton.

Additions	pmoles of fMet-tRNA bound or of fMet-puromycin extracted			
	Experiment 1		Experiment 2	
	−puro	+puro	−Sm	+Sm
None	6.90	7.59	1.08	0.539
Me_2SO 4% (final conc.)	7.62	8.30	2.45	1.16
Me_2SO 4% + thiostrepton (0.005 mM)	7.69	8.8	1.67	0.9

The incubation mixture (50 μl) contained: Tris (pH 7.5), 50 mM; Mg acetate, 5 mM; NH_4Cl, 80 mM; GTP, 1 mM; ribosome, 1 A_{260} unit; IF-1, 3.8 μg. Experiment 1: poly AUG, 0.15 A_{260} unit; IF-2, 2.45 μg; ^{3}H-fMet-tRNA (13.5 pmoles/A_{260} unit), 0.75 A_{260} unit. Incubation 10 min at 37°. Where indicated 100 μl of 70 mg % puromycin were added after incubation, and after 5 min at 37° the samples were diluted with 1 ml of ethyl acetate solution (pH 5) 0.1 M, and puromycin extracted according to Leder and Bursztyn, Biochem. Biophys. Res. Commun. 25 (1966) 233. Experiment 2: ApUpG, 0.05 A_{260} unit; IF-2, 15.6 μg; ^{3}H-fMet-tRNA (24.7 pmoles/A_{260} unit), 0.98 A_{260} unit. Incubation 20 min at 37°. Where indicated 1 μg of streptomycin was added and a further incubation of 10 min at 37° was carried out.

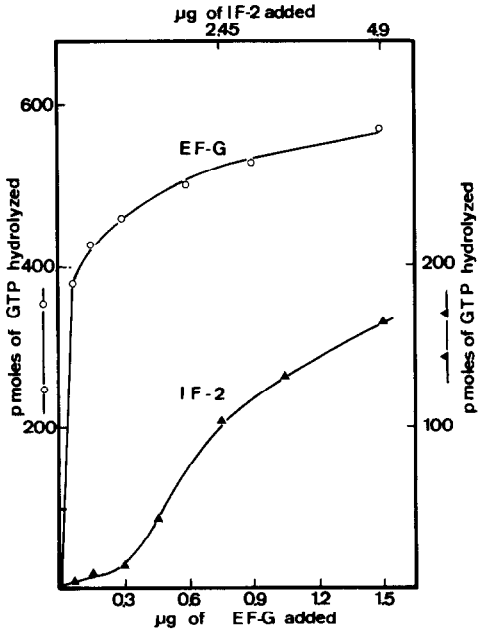

Fig. 1. GTP hydrolysis as a function of factor concentrations. The incubation mixture (25 μl) contained: Tris (pH 7.4), 50 mM; NH_4Cl, 80 mM; Mg acetate, 10 mM; β-mercaptoethanol, 7 mM; 70 S ribosomes, 1 A_{260} units; ^{32}P-GTP, 720 pmoles (specific activity: 2.14–0.88 Ci/mmole); IF-2 and EF-G, as indicated. GTP hydrolysis was determined as already described [5]. Incubation 10 min at 37°.

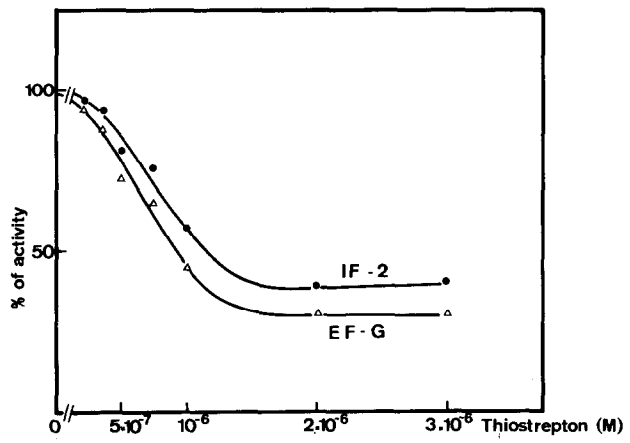

Fig. 2. Thiostrepton inhibition of EF-G and IF-2 mediated GTPase. Same conditions as in fig. 1, except for IF-2, 2.45 μg; EF-G, 0.3 μg; and thiostrepton in Me_2SO solution, as indicated. The final Me_2SO concentration was always kept inferior to 2%; under these conditions no appreciable GTPase stimulation occurs. Inhibition was calculated as compared to the sample without Me_2SO. The molecular weight of thiostrepton was taken as 1616 according to B. Anderson, D.C. Hodgkin and M.A. Wiswanit, Nature 225 (1970) 233.

3.2. *Inhibition of IF-2- and EF-G-dependent GTPase by thiostrepton*

Fig. 1 shows IF-2 and EF-G-dependent ribosomal GTPase as a function of factor concentrations; very little GTPase is exhibited by factor alone or by ribosome alone. It should be noted that at low IF-2 concentrations the curve is not linear. Fig. 2 shows the inhibition of the two GTPases as a function of thiostrepton concentration: both GTPases are inhibited to about the same extent by approx. 1 molar equivalence of thiostrepton to ribosome. Me_2SO, on the other hand, at a conc. above 2% highly stimulates both GTPases to about 100% (table 2). IF-1, as already noted, slightly inhibits the IF-2-dependent GTPase; the inhibition is more pronounced at 8% Me_2SO.

Table 2
Influence of Me_2SO on the IF-2- and EF-G-dependent GTPase.

Additions	Me_2SO (final concentration) 0	4%	8%
	(pmoles P_i liberated)		
Rib.	11.8	11	14
IF-2	0	0	0
Rib. + IF-2	70	140	164
Rib. + IF-2 + IF-1	64	–	103
EF-G	12	12	12
Rib. + EF-G	50	110	103

Same conditions as in fig. 1 except for the amount of IF-1 = 3.6 μg.

Table 3
Relation between IF-2- and EF-G-dependent GTPase.

Addition	P_i liberated (pmoles)
Rib. + EF-G + fus.	162
Rib. + EF-G	785
Rib. + IF-2 + fus.	133
Rib. + IF-2	133
Rib. + IF-2 + fus. + EF-G	203

Same conditions as in fig. 1, except that where indicated 1 mM of sodium fusidate was added to the incubation mixture. IF-2-dependent GTPase corresponds to 41 pmoles of P_i liberated (after subtraction of EF-G-dependent GTPase).

3.3. *Inhibition of IF-2-dependent GTPase by the complex: EF-G–GDP–fusidic acid*

Since thiostrepton is believed to inactivate the EF-G binding site, and inhibits the IF-2-dependent GTPase, suggesting that the antibiotic binding site is involved in this GTPase, we further investigated whether the elongation factor-dependent GTP binding site on the 50 S subunit was involved in the initiation factor-dependent GTPase.

Table 4
Inhibition by EF-G of EF-T-dependent Phe-tRNA binding.

Additions	pmoles of Phe-tRNA bound	
	+GTP	+GMP-PCP
Rib. + fus.	0.34	0.26
Rib. + fus. + EF-T	0.97	0.50
Rib. + fus. + EF-G	0.30	0.24
Rib. + fus. + EF-G + EF-T	0.64	0.25

The reaction mixture (50 μl) contained: Tris (pH 7.5), 50 mM; NH_4Cl, 80 mM; Mg acetate, 5 mM; GTP, 1 mM; ribosomes, 1 A_{260} unit; IF-2, 2.5 μg; sodium fusidate, 1.2 mM; ^{14}C-Phe-tRNA (13,000 cpm/A_{260} unit), 1 A_{260} unit; where indicated: EF-G, 14.5 μg, and EF-T, 2 μg. A preincubation of 5 min was carried out at 25° without EF-T nor Phe-tRNA in 40 μl, then the two products were added, the samples were completed to 50 μl and further incubated 20 min at 25°.

The EF-G site was therefore occupied by formation of a stable complex: EF-G–GDP–fusidic acid–ribosome, and the initiation factor-dependent ribosomal GTPase was investigated. Fusidic acid, while inhibiting the elongation factor-dependent GTPase, does not inhibit, as previously shown, the initiation factor-dependent GTPase; however, addition of EF-G strongly inhibits this last GTPase. Table 3 shows that in the presence of fusidic acid (after subtracting the residual EF-G-dependent GTPase from the initiation factor-dependent GTPase), only 40 pmoles of phosphate are liberated from GTP, whereas in the absence of EF-G, 133 pmoles are liberated. In the absence of fusidic acid, EF-2-dependent GTPase is somewhat inhibited by EF-G at saturating concentrations, but to a much lesser extent than when fusidic acid is present.

3.4. *Inhibition of EF-T-stimulated Phe-tRNA binding by the complex: GMP–PCP–EF-G–ribosome–fusidic acid*

It has been postulated that the EF-G binding site was the recognition site, A [14]; we therefore investigated whether the the stable complex: EF-G–GTP (or GDP)–ribosome–fusidic acid would prevent the binding of aminoacyl-tRNA. In order to avoid any residual polymerization, the experiment was also performed in the presence of GMP-PCP, a non-hydrolyzable analog of GTP.

As can be seen from table 4 the binding of Phe-tRNA is strongly inhibited by the presence of fusidic acid and of EF-G, confirming that the binding site of this factor overlaps the A site.

4. Discussion

Blocking EF-G binding site (either by thiostrepton or by the complex EF-G–GDP–fusidic acid) prevents binding of Phe-tRNA–EF-Tu–GTP to the 50 S ribosome subunit, suggesting that the elongation factors (EF-G and EF-T) binding site could be correlated to the recognition site, A; moreover, such a blocking inhibits the initiation factor-dependent GTPase.

One can therefore conclude that, although the ribosomal attachment site for the elongation factors is on the 50 S subunit [10–13] and that for initiation factors on the 30 S subunit [19–21], a topographical relationship exists together with a certain degree of interdependence between the target of IF-2 on the 70 S ribosomal initiation complex and the reactive site of elongation factors.

One of the explanations would be that IF-2-dependent GTP hydrolysis is correlated with the exit of this factor from the ribosome which would take place on a site on the 50 S identical with, or overlapping, the recognition site A. Our data are consistent with the hypothesis of Benne and Voorma [22] that when GTP is replaced by its non-hydrolyzable analog, GMP-PCP, the initiation factor cannot be removed from the ribosome and blocks the A site, thus explaining the lack of puromycin reactivity of the bound fMet-tRNA in the presence of the analog. The observed inhibition of Phe-tRNA binding by IF-2 is also in favor of this hypothesis [23].

Another explanation not exclusive of the former, is that GTP hydrolysis results from a conformational change of one of the ribosomal subunits, leading to the creation of more affinity sites for GTP. This could be caused by interaction between elongation factor, EF-G, and the 50 S ribosome subunit, or by interaction of initiation factor, IF-2, and the 30 S subunit. It has already been shown that the presence of 30 S subunits enhances the peptidyl transferase activity carried by the 50 S [24]. It is also not excluded that IF-2 mediated GTP hydrolysis takes place on the 30 S subunit, but attachment of an antibiotic, or of EF-G in a stable way, on the 50 S subunit (as by a GDP–fusidic acid complex) could interfere with the 30 S subunit conformational change responsible for GTP hydrolysis. The fact that a solvent, such as Me_2SO stimulates the factor-dependent GTPase, and that IF-1 somewhat prevents this stimulation, would suggest that a conformational change of ribosome is involved in GTP hydrolysis. In any case, our experiments indicate that the factor-mediated reaction has a long range effect on the subunit complementary to the one at the level of which this reaction occurs.

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