Proc. Nat. Acad. Sci. USA
Vol. 70, No. 7, pp. 1939–1942, July 1973

Effect of Thiostrepton on Recycling of *Escherichia coli* Initiation Factor 2

(L7, L12/50S subunit/fMet-puromycin/GTP/GMPPCP)

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Communicated by Severo Ochoa, April 17, 1973

ABSTRACT The recycling of initiation factor 2, which requires both GTP and the 50S subunit and involves GTP hydrolysis, appears to be blocked by thiostrepton. However, this antibiotic has little or no effect on the AUG-directed ribosomal binding promoted by initiation factor 2 of fMet-tRNA under conditions that do not permit optimal recycling of the factor. Evidence is presented suggesting that the 50S ribosomal proteins L7 or L12, which are required for the function of the chain elongation factors Tu and G and the accompanying hydrolysis of GTP, are also involved in recycling of initiation factor 2. Although both 5′-guanylylmethylene diphosphonate and thiostrepton seem to block recycling of initiation factor 2, fMet-tRNA bound to the ribosomes in the presence of GTP and thiostrepton can react with puromycin, whereas that bound in the presence of 5′-guanylylmethylene diphosphonate and the antibiotic cannot. It is proposed that initiation factor 2 may interact with two nonidentical ribosomal sites during polypeptide chain initiation.

The peptide antibiotic, thiostrepton, binds to the 50S ribosomal subunit and thereby impairs the ribosomal functions dependent on elongation factors G (EF-G) and Tu (EF-Tu), including the hydrolysis of GTP (1–4). These ribosomal functions, in addition, require the presence of the 50S ribosomal proteins L7 or L12 or both (5–7). It has recently been reported that thiostrepton causes partial inhibition of the uncoupled GTP hydrolysis dependent on the *Escherichia coli* initiation factor 2 (IF-2) and ribosomes (8).

The results of the present study suggest that the recycling of IF-2, i.e., the ability of this factor to promote several rounds of initiation, a process that requires both GTP and the 50S subunit (9–12), is blocked by thiostrepton. It also appears that ribosomes or 50S subunits deficient in L7 and L12 have a reduced ability to support the recycling of IF-2. This impaired ribosomal function is partially restored by addition of L7 and L12.

MATERIALS AND METHODS

The pH of all buffers was measured at 25°; Mg^{2+} was added as magnesium acetate.

Ribosomes and Ribosomal Subunits. Ribosomal subunits and ribosomes, washed twice with buffers containing 1 M NH_4Cl, were prepared from *E. coli* Q13 essentially as described (9). Ribosomes and 50S subunits, deficient in L7 and L12, were prepared by treating 426 and 81 A_{260} units, respectively, with 40% ethanol–1 M NH_4Cl in a final volume of 2.2 ml as described by Brot *et al.* (6). The treated preparations were dialyzed against a buffer containing 20 mM Tris·HCl (pH 7.4)–50 mM NH_4Cl–10 mM Mg^{2+}. Preparations not treated with ethanol–NH_4Cl were used as controls.

Other Preparations. IF-2 (PC IF-2), prepared from 1 M NH_4Cl washes of *E. coli* Q13 ribosomes by $(NH_4)_2SO_4$ fractionation and phosphocellulose chromatography (9), was used in experiments requiring large amounts of this factor. For experiments involving the use of limiting amounts of IF-2, this factor was purified from 0.5 M NH_4Cl washes of *E. coli* K12λ ribosomes by ammonium sulfate fractionation, followed by successive gradient elution from phosphocellulose and quaternary aminoethyl (QAE) Sephadex columns (Mazumder, R., unpublished). This preparation is referred to as QAE IF-2. Initiation factor 1 (IF-1) was prepared from *E. coli* K12λ essentially by the method described (13) except that the heating step was omitted. Frozen *E. coli* K12λ cells (late logarithmic phase) were obtained commercially from General Biochemicals. Measurement of the ribosomal binding of f[^{14}C]Met-tRNA and of the synthesis of f[^{14}C]Met-puromycin have been described (9). 10^{-2} M thiostrepton in Me_2SO and a sample containing a mixture of L7 and L12 proteins were generously provided by Dr. H. Weissbach, Roche Institute of Molecular Biology, Nutley, N.J.

RESULTS

Effect of thiostrepton on recycling of IF-2

fMet-tRNA Binding to Ribosomes. As shown in Fig. 1, relatively low concentrations of thiostrepton strongly inhibit the binding of fMet-tRNA under conditions that permit recycling of IF-2, i.e., when GTP is present and the amount of IF-2 used is limiting with respect to ribosomes. It may be seen that this inhibition occurs over a rather narrow range of thiostrepton concentrations. Maximal inhibition was obtained around 8.3×10^{-6} M when the ribosome concentration was about 4.4×10^{-6} M. It should also be noted that the inhibition is not complete, since that amount of fMet-tRNA binding that is stoichiometric with IF-2 is unaffected by thiostrepton (see below). Although the experiment shown in Fig. 1 was done by first incubating ribosomes at 37° with various concentrations of thiostrepton, later studies showed that preincubation at 37° was not a prerequisite for obtaining inhibition. The remaining experiments in this paper were, therefore, done by addition of the antibiotic directly to ribosome-containing reaction mixtures at 0° before addition of IF-2.

If thiostrepton only blocks the additional rounds of initiation due to recycling of IF-2, but not the ribosomal binding of fMet-tRNA stoichiometric with IF-2, it may be predicted that the fMet-tRNA binding observed in the presence of GTP, thiostrepton, and a constant amount of ribosomes should be proportional to the amount of IF-2 added, over a *wider* range of IF-2 concentrations than when the antibiotic is

Abbreviations: Me_2SO, dimethylsulfoxide; GMPPCP, 5′-guanylylmethylene diphosphonate; IF-1, IF-2, EF-G, and EF-Tu are the accepted abbreviations for initiation factors F_1, F_2, and elongation factors G and Tu, respectively (17).

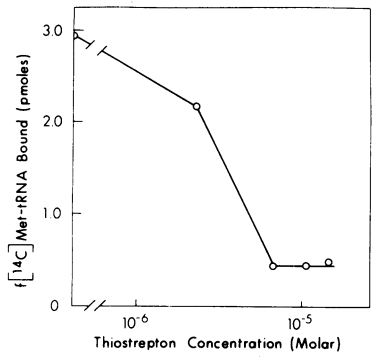

FIG. 1. Effect of increasing concentrations of thiostrepton on the ribosomal binding of fMet-tRNA in the presence of GTP and limiting amounts of IF-2. Ribosomes (22 A_{260} units) were incubated at 37° for 5 min either without or with the indicated concentrations of thiostrepton in a buffer containing 10 mM Mg^{2+}–3 mM Tris·HCl (pH 7.4)–30 mM NH_4Cl–0.06 mM dithiothreitol–6% glycerol. Me_2SO concentration (0.25%, v/v) was the same in each incubation. Volume was 0.12 ml. 10-μl aliquots were then added to reaction mixtures (final volume, 0.05 ml) containing 50 mM Tris·HCl (pH 7.3), 3 mM Mg^{2+}, 100 mM NH_4Cl, 5 mM dithiothreitol, 0.2 mM GTP, 0.058 A_{260} units of AUG, 0.3 μg of QAE IF-2, 0.45 μg of IF-1. 20 pmol of f[^{14}C]Met-tRNA was added last, and incubation was for 15 min at 0°. Ribosomal binding of f[^{14}C]Met-tRNA (specific activity, 360 cpm/pmol) was assayed by Millipore filtration. Blank without added factors (0.11 pmol) has been subtracted.

omitted. The results summarized in Table 1 demonstrate that this is indeed the case. When the concentration of IF-2 is varied by a factor of 10 with a constant amount of ribosomes, a proportional binding of fMet-tRNA is observed only when thiostrepton is present. It appears, therefore, that the addition of thiostrepton converts a catalytic system into a stoichiometric one. It may also be noted that, at high concentrations of IF-2, the percent inhibition of fMet-tRNA binding by thiostrepton is relatively low, since smaller amounts of ribosomes are available to bind fMet-tRNA utilizing IF-2 released by recycling (see also Table 2).

It has been reported that, unlike GTP, 5′-guanylylmethylene diphosphonate (GMPPCP) does not permit the recycling of IF-2, since this factor is released from 70S initiation complexes formed in the presence of GTP, but not from those formed in the presence of GMPPCP (refs. 11, 14, and especially 12*). As is to be expected, the ribosomal binding of fMet-tRNA in the presence of GMPPCP is only slightly inhibited by thiostrepton (Table 2).

fMet-tRNA Binding with Ribosomal Subunits. It has been reported that both GTP and the 50S subunit are required for the recycling of limiting amounts of IF-2 (9). Similar observations have been made in other laboratories (10–12, 14). As mentioned previously, thiostrepton is an inhibitor that is specific for the 50S subunit. The results summarized in Table 3 clearly indicate that, as expected, thiostrepton has little or no effect on the IF-2-promoted binding of fMet-tRNA on the 30S subunit. In contrast, fMet-tRNA binding, due to recycling

* See also Fakunding, J., Traugh, J. A., Traut, R. R. & Hershey, J. W. B. (1972). "Interaction of enzymically phosphorylated initiation factor F_2 with ribosomes," *Fed. Proc.* **31**, 410.

TABLE 1. *Proportionality between IF-2 concentration and ribosomal binding of fMet-tRNA in the presence of thiostrepton and GTP*

Exp. no.	IF-2 additions (μg)	Thiostrepton	f[^{14}C]Met-tRNA bound (pmol)	Inhibition by thiostrepton (%)
1	40.0	Absent	6.75	—
	40.0	Present	5.80	14
	4.0	Absent	1.75	—
	4.0	Present	0.64	63
2	40.0	Absent	6.30	—
	40.0	Present	3.93	37
	4.0	Absent	2.09	—
	4.0	Present	0.38	82

Reaction mixtures (0.050–0.060 ml) contained: 50 mM Tris·HCl buffer (pH 7.3), 4 mM Mg^{2+}, 116 mM NH_4Cl, 5 mM dithiothreitol, 1% glycerol, 0.058 A_{260} units of AUG, 0.2 mM GTP, PC IF-2 as indicated; 0.45 μg of IF-1 (in Exp. 2 only), 20 pmol of f[^{14}C]Met-tRNA, 1.83 A_{260} units of ribosomes, and, where indicated, 10^{-5} M thiostrepton. All tubes contained 0.1% (v/v) Me_2SO. f[^{14}C]Met-tRNA was added last, and incubation was for 45 min at 0°. Blank without added factors (about 0.1 pmol) has been subtracted. Binding was measured by Millipore filtration.

of IF-2, which is observed upon addition of GTP and the 50S subunit, is strongly inhibited by the antibiotic.

Effect of thiostrepton on fMet-puromycin synthesis with GTP or GMPPCP

As shown in Table 2, thiostrepton does not block the puromycin reactivity of fMet-tRNA bound to ribosomes in the presence of GTP. Similar observations have been previously made with this antibiotic (8) as well as with siomycin (15). It should also be noted that the low puromycin reactivity of fMet-tRNA bound to ribosomes in the presence of GMPPCP is not increased by thiostrepton.

Since, like GMPPCP, thiostrepton seems to block the recycling of IF-2, the expectation is that, for a given amount of this factor, the amount of fMet-tRNA bound to ribosomes in the presence of thiostrepton should be about the same with GTP or GMPPCP. However, as shown in Table 2, the ribosomal binding of fMet-tRNA with GTP is about twice that with GMPPCP under these conditions. The possible significance of these observations is discussed below.

Effect of ethanol–NH_4Cl treatment of ribosomes on recycling of IF-2: Role of L7 and L12

It has been reported that, after treatment with 40% ethanol–1 M NH_4Cl, ribosomes become deficient in proteins L7 and L12 (5, 6). Since the 50S subunit is required for recycling of IF-2, it was of interest to see the effect of such a treatment on the ability of ribosomes or 50S subunits to support this recycling. It may be seen from the results given in Tables 4 and 5 that, as compared to controls, treated preparations are only about 40% as active for fMet-tRNA binding. Addition of L7 and L12 to treated ribosomes or 50S subunits results in a stimulation of binding to a value of about 60% that of controls. In contrast, addition of L7 and L12 to control ribosomes has

TABLE 2. *Effect of thiostrepton on synthesis of fMet-puromycin in the presence of GTP or GMPPCP*

Nucleotide added	Thiostrepton	f[^{14}C]-Met-tRNA bound* (pmol)	f[^{14}C]-Met-puromycin formed† (pmol)	Puromycin reactivity (%)
GTP	Absent	7.35	6.70	91
GTP	Present	6.25	5.20	83
GMPPCP	Absent	3.38	0.31	9
GMPPCP	Present	3.26	0.21	6

Reaction mixtures (0.050 ml) contained: 50 mM Tris·HCl buffer (pH 7.3), 4 mM Mg^{2+}, 116 mM NH_4Cl, 5 mM dithiothreitol, 0.2 mM GTP or GMPPCP, 1% glycerol, 0.058 A_{260} units of AUG, 20 pmol of f[^{14}C]Met-tRNA, 40 μg of PC IF-2, 1.83 A_{260} units of ribosomes, and 10^{-5} M thiostrepton, when present. All tubes contained 0.1% (v/v) Me_2SO. Reaction was started by addition of f[^{14}C]Met-tRNA. After 15 min at 0°, 5 μl of 10 mM puromycin was added to one set of tubes, while another identical set received 5 μl of water. After further incubation for 30 min at 0°, the amount of fMet-tRNA bound and its puromycin reactivity was measured.

* Net values (blanks without added IF-2 subtracted). The blanks averaged 0.14 pmol.

† Net values (blanks without puromycin subtracted). The blanks averaged 0.32 pmol.

virtually no effect (Table 4) and is actually inhibitory when added to control 50S subunits (Table 5). The reason why full activity is not restored upon recombination of treated ribosomes with L7 and L12 is not clear.

DISCUSSION

The relatively low puromycin reactivity of fMet-tRNA, bound to ribosomes in the presence of GMPPCP, is now believed to be due to the fact that in the absence of GTP hydrolysis, IF-2 cannot recycle and, instead, remains attached to ribosomes. Under these conditions, IF-2 is thought to block a ribosomal site that would otherwise be accessible to an incoming aminoacyl-tRNA or its analog puromycin (16). The present study shows that thiostrepton, an antibiotic specific for the 50S subunit, interferes with the recycling of IF-2 even though GTP is present. A likely explanation of this observation is that thiostrepton specifically blocks a 50S subunit and IF-2-dependent ribosomal GTPase that is essential for ejecting this factor from the ribosome. This explanation is consistent with the report (8) that thiostrepton inhibits an IF-2-dependent ribosomal GTPase. From the above discussion, it may be predicted that, as with GMPPCP,

TABLE 3. *Effect of thiostrepton on fMet-tRNA binding with ribosomal subunits and GTP*

Exp. no.	Subunits: 30S (A_{260} units)	Subunits: 50S (A_{260} units)	Thiostrepton (10^{-5} M)	f[^{14}C]Met-tRNA bound (pmol)
1	0.6	None	Absent	0.28
	0.6	None	Present	0.26
	0.6	1.19	Absent	2.91
	0.6	1.19	Present	0.59
2	0.6	None	Absent	1.56
	0.6	None	Present	1.68
	0.6	1.19	Absent	6.50
	0.6	1.19	Present	2.55

Conditions similar to that of Table 1 with 0.3 μg of QAE IF-2 (Exp. 1) or 20 μg or PC IF-2 (Exp. 2); 0.45 μg of IF-1 (Exp. 1) and ribosomal subunits, as indicated. Incubation was at 0° for 15 min (Exp. 1) or for 30 min (Exp. 2). Blanks without added factors, averaging about 0.14 pmol, have been subtracted.

TABLE 4. *Effect of L7 and L12 on the ribosomal binding of fMet-tRNA with GTP and either control or ribosomes treated with ethanol–NH_4Cl*

Ribosomes added	L7 + L12 addition (μg)	f[^{14}C]Met-tRNA bound (pmol)	Relative activity
Control	None	5.07	100
Control	10	5.14	101
Treated	None	1.81	35
Treated	10	2.94	58

Reaction mixtures (0.05 ml) contained: 50 mM Tris·HCl buffer (pH 7.3), 6 mM Mg^{2+}, 110 mM NH_4Cl, 5 mM dithiothreitol, 0.2 mM GTP, 0.058 A_{260} units of AUG, 20 pmol of f[^{14}C]Met-tRNA, 8 μg of PC IF-2, 2.0 A_{260} units of either control or ethanol–NH_4Cl-treated ribosomes, and, where indicated, 10 μg of a mixture of L7 + L12. Ribosomes were kept at 37° for 2 min, with or without L7 + L12, before other additions. f[^{14}C]Met-tRNA was added last. Incubation was for 30 min at 0°. Blanks minus IF-2 (with or without L7 + L12), averaging 0.24 pmol, have been subtracted.

TABLE 5. *Effect of L7 and L12 on the ribosomal binding of fMet-tRNA with GTP and either control or 50S subunits treated with ethanol–NH_4Cl*

Exp. no.	Additions	f[^{14}C]-Met-tRNA bound (pmol)	Stimulation by 50S subunits (pmol)	Relative activity
1	30S	0.45	—	—
	30S + control 50S	2.97	2.52	100
	30S + treated 50S	1.49	1.04	41
	30S + treated 50S + L7 + L12	1.96	1.51	60
2	30S	0.58	—	—
	30S + control 50S	2.48	1.90	100
	30S + control 50S + L7 + L12	1.78	1.20	63
	30S + treated 50S	1.55	0.97	51
	30S + treated 50S + L7 + L12	1.97	1.39	73

Reaction mixtures (0.06 ml) contained: 50 mM Tris·HCl buffer (pH 7.3), 5.4 mM Mg^{2+}, 112 mM NH_4Cl, 4 mM dithiothreitol, 0.058 A_{260} units of AUG, 20 pmol of f[^{14}C]Met-tRNA, 0.3 μg of QAE IF-2, 0.45 μg of IF-1, 0.6 A_{260} units of 30S subunits, 1.2 A_{260} units of control or ethanol–NH_4Cl-treated 50S subunits, when present, and 10 μg of a mixture of L7 and L12, when present. 50S subunits were kept at 37° for 2 min with or without L7 + L12 before other additions. f[^{14}C]Met-tRNA was added last. Incubation was for 30 min at 0°.

a *low* amount of fMet-puromycin synthesis should also be observed with GTP plus thiostrepton. In either case, IF-2 can not recycle and should, therefore, remain associated with and block a ribosomal site whose accessibility is important for the puromycin reaction. The *high* puromycin reactivity of fMet-tRNA bound to ribosomes in the presence of GTP and thiostrepton is, therefore, surprising, especially since the antibiotic does not increase the low amount of fMet-puromycin synthesis observed with GMPPCP (Table 2). This apparent discrepancy could be explained by assuming that IF-2 may block nonidentical ribosomal sites when initiation complexes are formed with GMPPCP on the one hand and with GTP plus thiostrepton on the other. The following model is proposed to explain the results obtained in this study. fMet-tRNA in a 70S initiation complex formed with GMPPCP shows a low puromycin-reactivity since IF-2, present in such a complex, blocks a ribosomal site (site "X") whose accessibility is essential for good reactivity with puromycin. The removal of IF-2 from site "X" requires GTP, and this removal is blocked by GMPPCP but *not* by thiostrepton. In contrast, the GTP-dependent removal of IF-2 from the other ribosomal site (site "Y") is blocked not only by GMPPCP but also by thiostrepton. However, there is little or no interference with fMet-puromycin synthesis when this ribosomal site "Y" is occupied by IF-2. The results suggest the possibility, therefore, that a part of the IF-2-dependent ribosomal GTPase (perhaps that related to removal of IF-2 from site "X") may be insensitive to thiostrepton. The observation (8) that ribosomal GTPase, dependent on IF-2, is only *partially* inhibited by thiostrepton, is in line with this suggestion.

The 50S ribosomal proteins L7 and L12 are involved in the ribosomal functions dependent on the elongation factors EF-G and EF-Tu (5, 6). The present study suggests that these proteins are also involved in the recycling of IF-2 dependent on the 50S subunit and GTP. It is very likely that, as for the EF-Tu- and EF-G-dependent GTPase, L7 and L12 are also required for optimal activity of a thiostrepton-sensitive ribosomal GTPase dependent on both IF-2 and the 50S subunit.

The observation that, in the presence of thiostrepton, the amount of fMet-tRNA bound to ribosomes with GMPPCP is only about 50% of that obtained with GTP (Table 2) is puzzling. Although alternative explanations are possible, the results are consistent with a model in which *two* IF-2 molecules (per 70S initiation complex) are unable to participate in additional rounds of initiation when complexes are formed under these conditions with GMPPCP. In contrast, a *single* IF-2 molecule (per 70S initiation complex) may not recycle when initiation complexes are formed with GTP and thiostrepton. In this connection, it is interesting to note that other investigators have made the observation that the amount of fMet-tRNA bound to 30S subunits with GMPPCP is about 50% of that obtained with GTP (10, 11). The data are again consistent with a model where the number of IF-2 molecules associated (perhaps weakly) with a 30S initiation complex formed with GMPPCP is twice that present in an analogous complex formed with GTP.

NOTE ADDED IN PROOF

It has recently been observed that the AUG-directed ribosomal binding of fMet-tRNA is inhibited much more by thiostrepton when the binding reaction is performed at 0° rather than at 25°.

I thank Dr. Severo Ochoa for his interest in this work and for help in the preparation of the paper, and Dr. Michael Zasloff for his critical reading of the manuscript.. Helpful discussions with Dr. Herbert Weissbach, Roche Institute of Molecular Biology, Nutley, N.J., is gratefully acknowledged. Miss Jaroslava Husa provided excellent technical assistance. I thank Miss Jennifer Chu for preparation of ribosomal subunits. This work was aided by Grants AM 01845, FR 05399, and GM 01234 from the National Institutes of Health, U.S. Public Health Service, and Grant P 558 from the American Cancer Society.

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