Proc. Nat. Acad. Sci. USA
Vol. 69, No. 10, pp. 2770–2773, October 1972

Initiation Factor 2-Dependent Ribosomal Binding of *N*-Formylmethionyl-Transfer RNA Without Added Guanosine Triphosphate

(30S-initiation complex/fMet-puromycin/recycling of IF-2/50S subunit/IF-1)

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Communicated by Severo Ochoa, July 21, 1972

ABSTRACT **Evidence is presented that suggests that GTP (or 5'-guanylylmethylene diphosphonate) is not essential for either formation of an AUG-directed, initiation factor IF-2-dependent initiation complex on the 30S subunit or for positioning of fMet-tRNA on the peptidyl site. However, recycling of limiting amounts of IF-2 requires both GTP and the 50S subunit. The formation of the complex IF-2–30S as an intermediate in polypeptide-chain initiation is suggested.**

It is generally agreed that, in prokaryotic cells, GTP (or its analogue, 5'-guanylylmethylene diphosphonate) is essential for the messenger-directed, initiation factor-dependent binding of fMet-tRNA to ribosomes (1). However, in eukaryotic cells, a supernatant factor promotes a GTP-independent, AUG-directed binding of fMet-tRNA to 40S ribosomal subunits (2, 3), and the bound aminoacyl-tRNA reacts directly with puromycin upon addition of 60S subunits. A factor-dependent, GTP-independent binding of fMet-tRNA to 40S ribosomal subunits was observed in reticulocyte extracts (4).

Evidence will be presented in this paper that, under suitable conditions, there is considerable AUG-directed binding of fMet-tRNA to 30S subunits in the absence of added GTP. The extent of this binding may be as high as 85% of that obtained when GTP is added. In contrast, both GTP and 50S subunits appear to be essential for the recycling of limiting amounts of initiation factor IF-2. Half or more of the fMet-tRNA in the ribosomal complex formed without added GTP reacts with puromycin. This suggests that the IF-2-promoted positioning of fMet-tRNA on the peptidyl site does not require added GTP. It is proposed that a binary complex, IF-2–30S, rather than a ternary complex, GTP–IF-2–fMet-tRNA, is an intermediate in polypeptide-chain initiation.

MATERIALS AND METHODS

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

Ribosomes and Ribosomal Subunits. Crude ribosomes from *Escherichia coli* Q13 were washed once overnight with 1 M NH_4Cl–20 mM Tris·HCl (pH 8.1)–5 mM Mg^{2+}–1 mM S_2 threitol. The washed ribosomal pellet was diluted to a concentration of about 300 A_{260} units/ml in a buffer containing 500 mM NH_4Cl–2 mM Mg^{2+}–20 mM Tris·HCl (pH 8.1)–0.5 mM S_2 threitol, layered on 5–30% sucrose gradients (50–55 ml) containing this buffer, and centrifuged in a Spinco SW25.2 rotor for 13.5 hr at 23,000 rpm. Fractions from these gradients were pooled to give 30S and 50S subunits. After the Mg^{2+} concentration was raised to 10 mM, the subunits were pelletted by overnight centrifugation at 50,000 rpm in a Spinco 65 rotor. The subunits were taken up in a small volume of the solution from which they were pelletted, and after addition of an equal volume of glycerol, the subunits were stored at −20° without freezing at a concentration of 150 A_{260} units/ml or higher. The subunits were dialyzed for 2.5 hr against a buffer containing 50 mM Tris·HCl (pH 7.3)–50 mM NH_4Cl–7.5 mM Mg^{2+}–0.5 mM S_2 threitol. The dialyzed subunits (70–90 A_{260} units/ml) were stored at 4°.

Other Preparations. The preparation referred to as IF-2 represents the second peak of activity that was eluted from phosphocellulose columns ahead of the IF-2b peak with a buffer (buffer B, ref. 5) containing 250 mM NH_4Cl–50 mM Tris·HCl (pH 7.4)–1 mM S_2 threitol. Fractions containing this peak of IF-2 activity were pooled, and brought to 70% saturation with ammonium sulfate. The suspension was centrifuged, and the precipitate was dissolved in a small

TABLE 1. *Binding of fMet-tRNA to 30S subunits without added GTP*

Experiment no.	Additions	f[^{14}C]Met-tRNA bound (pmol)
1	30S	0.76
	30S, IF-2	4.72 (3.96)
	30S, IF-2, GTP	5.67 (4.91)
2	30S	0.30
	30S, IF-2	3.92 (3.62)
	30S, IF-2, GTP	4.48 (4.18)
	30S, IF-2 (no AUG)	0.23

Samples had the following composition: Experiment 1, Tris·HCl (pH 7.3), 64 mM; Mg^{2+}, 5.2 mM; NH_4Cl, 130 mM; S_2 threitol, 0.2 mM; glycerol, 0.5%; dialyzed 30S subunits, 0.58 A_{260} unit; dialyzed IF-2 (when present), 43 μg; f[^{14}C]Met-tRNA, 10 pmol; volume, 0.05 ml. Experiment 2, Tris·HCl (pH 7.3), 64 mM; Mg^{2+}, 5 mM; NH_4Cl, 125 mM; S_2 threitol, 0.25 mM; dialyzed 30S subunits, 0.43 A_{260} unit; dialyzed IF-2 (when present), 50 μg; f[^{14}C]Met-tRNA, 34 pmol; volume, 0.06 ml; GTP, 0.01 μmol; AUG, 0.058 A_{260} unit, added as indicated. The reaction was started by the addition of 30S subunits. Incubation was for 15 min at 0°.

Abbreviations: S_2 threitol, dithiothreitol; IF-1, IF-2, and EF-T are the accepted abbreviations for initiation factors F_1 and F_2 and elongation factor T, respectively.

volume of buffer B, made 50% with respect to glycerol, and stored at −20° without freezing. Aliquots of this IF-2 preparation were dialyzed before use for 4–5 hr against a buffer containing 100 mM NH_4Cl–20 mM Tris·HCl (pH 7.3)–0.5 mM S_2 threitol. IF-1 was purified by DEAE-cellulose and carboxymethyl (CM)-cellulose chromatography by Mr. S. Sabol of this laboratory. f[^{14}C]Met-tRNA (330–400 cpm/pmol) was prepared from crude *E. coli* tRNA (7); contaminating nucleotides were removed by gel filtration on Sephadex G-50. AUG was purchased from Miles Laboratories.

Ribosomal binding of f[^{14}C]Met-tRNA and f[^{14}C]Met-puromycin synthesis were measured essentially as described (5). The binding reaction was stopped by the addition of 2 ml of an ice-cold buffer containing 100 mM NH_4Cl–5 mM Mg^{2+}–50 mM Tris·HCl (pH 7.3). The Millipore filters were washed three times with 2.0 ml of this buffer. For study of fMet–puromycin formation, 1.0 ml of 0.1 M sodium acetate buffer (pH 5.4) was added to stop the reaction. The mixture was extracted with 1.5 ml of ethyl acetate for 2 min with a mixer. The radioactivity present in 1.1 ml of ethyl acetate layer was counted in Bray's solution. The results are expressed per 1.5 ml of ethyl acetate phase.

RESULTS

fMet-tRNA binding

It may be seen in Table 1 that, with large amounts of IF-2, there is good AUG-dependent binding of fMet-tRNA to the 30S ribosomal subunit at 0° in the absence of added GTP.

It was of interest to study the effect of the 50S subunit in the absence or presence of GTP. The results are summarized in Table 2. With large amounts of IF-2 (experiment 1), even in the presence of GTP, there is only a slight stimulation of binding by the 50S subunit. GTP stimulates binding only about 1.5-fold, even when both ribosomal subunits are present. However, the results are quantitatively different when IF-2 is made limiting by decreasing its concentration to one-fourth while increasing the concentration of subunits about 2-fold (experiment 2). Under these conditions, the cooperativity between the 50S subunit and GTP is clearly seen, for the 50S subunit increases the binding (about 4-fold) only when GTP is present. Similarly, the stimulation of binding by GTP is greater than 10-fold provided that the 50S subunit is also added.

TABLE 2. *Cooperativity between GTP and the 50S subunit for recycling of IF-2*

Experiment no.	Subunits 30S (A_{260} units)	Subunits 50S (A_{260} units)	IF-2 additions (μg)	GTP added (mM)	f[^{14}C]Met-tRNA bound* (pmol)
1	0.37	None	50.0	None	2.68
	0.37	None	50.0	0.16	3.08
	0.37	0.73	50.0	None	2.08
	0.37	0.73	50.0	0.16	3.65
2	0.68	None	12.5	None	0.60
	0.68	None	12.5	0.16	1.03
	0.68	1.45	12.5	None	0.33
	0.68	1.45	12.5	0.16	4.41

Samples (65 μl) contained: Tris·HCl (pH 7.3), 65 mM; Mg^{2+}, 5 mM; NH_4Cl, 125 mM; S_2 threitol, 0.25 mM; AUG, 0.058 A_{260} unit; f[^{14}C]Met-tRNA, 34 pmol; other additions as indicated. Reaction was started by addition of 30S subunits (or 30S immediately followed by 50S). Incubation was for 15 min at 0°.

* Net values (blanks with no added factors subtracted). Blanks averaged 0.2, 0.1, 0.1, and 0.1 pmol for 30S, 30S + GTP, 30S + 50S, and 30S + 50S + GTP, respectively.

TABLE 3. *Reactivity of fMet-tRNA bound to ribosomal subunits with puromycin without added GTP*

Experiment no.	f[^{14}C]Met-tRNA bound (pmol)	f[^{14}C]Met-puromycin formed* (pmol)	Puromycin reactivity (%)
1	2.13†	0.21	10
1a‡	3.47†	3.17	92
2	1.79†	0.44	24
3	0.39	0.25	64
4	1.74	0.94	54

* Net values (blanks without puromycin subtracted). The blanks were 0.12, 0.14, 0.02, and 0.02 for experiments 1–4, respectively.

† Net values (blanks without added factors subtracted). The blanks were 0.21 and 0.23 for experiments 1 and 2, respectively.

‡ GTP, 0.16 mM added.

Experiment 1. Samples (final volume, 65 μl) contained the following final concentrations: Tris·HCl (pH 7.3), 60 mM; Mg^{2+}, 4.8 mM; NH_4Cl, 123 mM; S_2 threitol, 0.23 mM; f[^{14}C]Met-tRNA, 34 pmol; AUG, 0.058 A_{260} unit; dialyzed 30S subunits, 0.37 A_{260} unit; dialyzed IF-2, 40 μg; IF-1, 2 μg; and GTP (in experiment 1*a*), 0.16 mM. The 30S subunits were added last, and the samples were incubated for 15 min at 0°. Dialyzed 50S subunits (0.73 A_{260} unit) were then added, and the mixture was incubated 5 min more 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 were measured. *Experiment 2.* Conditions were essentially the same as in experiment 1, with f[^{14}C]Met-tRNA, 40 pmol; dialyzed 30S subunits, 0.43 A_{260} unit; dialyzed 50S subunits, 0.96 A_{260} unit, and dialyzed IF-2, 46 μg; final volume, 0.07 ml. *Experiment 3.* Before gel filtration: Samples (final volume, 0.54 ml) contained the following final concentrations, Tris·HCl (pH 7.3), 65 mM; Mg^{2+}, 5.2 mM; NH_4Cl, 133 mM; S_2 threitol, 0.25 mM; AUG, 0.52 A_{260} unit; f[^{14}C]Met-tRNA, 326 pmol; dialyzed IF-2, 468 μg; IF-1, 18 μg; dialyzed 30S subunits, 3.44 A_{260} units. Incubation was for 15 min at 0° after addition of the 30S subunits. Dialyzed 50S subunits, 6.89 A_{260} units, were then added, and the mixture was incubated 15 min more at 0°. Binding was measured in a 20-μl aliquot by Millipore filtration. The remaining solution was applied to a Sephadex G-200 column (0.65 × 20 cm), previously equilibrated with buffer containing 50 mM Tris·HCl (pH 7.3)–100 mM NH_4Cl–5 mM Mg^{2+}–0.5 mM S_2 threitol. Fractions in the void volume were pooled to give about 0.7 ml of solution. After gel filtration: 2 hr later, 0.2-ml aliquots of the pooled fractions were incubated for 30 min at 0° with either 15 μl of water or 15 μl of 10 mM puromycin. Binding and fMet–puromycin formation were measured as described. *Experiment 4.* Conditions were essentially the same as for experiment 3, with the following exceptions: (*a*) The Sephadex column was equilibrated and eluted with buffer containing 50 mM Tris·HCl (pH 7.0)–100 mM NH_4Cl–7 mM Mg^{2+}–0.5 mM S_2 threitol; (*b*) Fractions in the void volume were pooled and 0.225-ml aliquots were *immediately* incubated either with 20 μl of water or 20 μl of 10 mM puromycin for 30 min at 0°.

TABLE 4. *Stimulation of the rate of fMet-tRNA binding to 30S subunits by GTP*

Incubation time (min)	GTP addition	f[^{14}C]Met-tRNA bound (pmol) (a)	(b)	Stimulation by GTP (a)	(b)
About 0.33	−	1.55	0.84		
About 0.33	+	6.15	3.98	×4.0	×4.7
2	−	3.87	2.69		
2	+	7.30	5.85	×1.9	×2.2
15	−	5.50	4.60		
15	+	8.92	6.08	×1.6	×1.3

Reaction mixtures (final volume, 0.28 ml) contained the following final concentrations: Tris·HCl (pH 7.3), 63 mM; Mg^{2+}, 5.8 mM; NH_4Cl, 110 mM; AUG, 0.23 A_{260} unit; f[^{14}C]Met-tRNA, 163 pmol; IF-1, 8 μg; dialyzed IF-2, 175 μg; dialyzed 30S, 1.7 A_{260} units; S_2 threitol, 0.25 mM; and GTP (when present), 0.18 mM. The reaction was started by either [values under (*a*)] addition of a mixture of IF-2 + 30S subunits in 50 mM Tris·HCl (pH 7.3), 50 mM NH_4Cl, 5 mM Mg^{2+}, 0.5 mM S_2 threitol (previously kept at 25° for 10 min, followed by 1 hr at 0°) or [values under (*b*)] by addition of 30S subunits alone. In the latter case, IF-2 and 30S subunits were treated separately, as described for the mixture of both, before use. Incubation was at 0°. At the indicated times, 75-μl aliquots were withdrawn into 3.0 ml of filtration buffer and binding was measured as described.

fMet-puromycin synthesis

The reactivity with puromycin of fMet-tRNA, bound in the presence of 30S and 50S subunits, but without added GTP, was tested in two ways (Table 3). The reaction mixture was either directly treated with puromycin (experiments 1 and 2) or passed through a Sephadex G-200 column before the addition of puromycin (experiments 3 and 4). Gel filtration has been used by Benne and Voorma (1) to increase the sensitivity to puromycin of fMet-tRNA bound to ribosomes, under the direction of bacteriophage MS2 RNA, in the presence of 5′-guanylylmethylene diphosphonate. It may be seen that the sensitivity to puromycin of fMet-tRNA bound to 30S and 50S subunits at 0° with AUG as template, without any added GTP or 5′-guanylylmethylene diphosphonate, is similarly increased when, as in the experiments of Benne and Voorma, the complex is freed of unbound components by gel filtration. Under these conditions (Table 3, experiments 3 and 4), more than half of the fMet-tRNA present in the isolated complex is reactive with puromycin. It should be pointed out that similar amounts of ribosome-bound complex (about 20 pmol; data not shown in Table 3) were formed before gel filtration in experiments 3 and 4. However, the recovery of complex (stable to Millipore filtration) after gel filtration was about 7.5% in experiment 3 and 25% in experiment 4. Since aliquots of the complex were filtered through Millipore filters after about 150 min in experiment 3 and after about 30 min in experiment 4, it would appear that the isolated complex is rather unstable.

Rate of fMet-tRNA binding

As mentioned previously, the amount of IF-2-dependent fMet-tRNA bound to 30S subunits (15 min, 0°) without added GTP approaches that bound when GTP is present (Table 1). However, rate analysis (Table 4) reveals that the rate of complex formation is faster in the presence of GTP. The reaction was started either with a mixture of 30S subunits and IF-2 or with 30S subunits alone as the last addition (see footnotes of Table 4). Since GTP stimulates the rate of binding in both cases, it appears that the rate-limiting event, which is subject to stimulation by GTP, is not the interaction of IF-2 with 30S subunits. However, the results also indicate that the rate of fMet-tRNA binding to the 30S subunits (with or without added GTP) is faster when the reaction is started with the mixture of IF-2 and 30S subunits than when the reaction is started with 30S subunits alone. This observation, as well as the IF-2-dependent binding of fMet-tRNA to the 30Ss subunit without added GTP, is consistent with the involvement of an IF-2–30S complex as an intermediate in polypeptide chain initiation.

DISCUSSION

On the basis of the results reported in this paper, the following sequence of reactions is proposed for the AUG-directed ribosomal binding of fMet-tRNA:

1. IF-2 + 30S $\rightleftharpoons$ [IF-2–30S]
2. [IF-2–30S] + fMet-tRNA + AUG $\overset{GTP}{\rightleftharpoons}$ [IF-2–30S–fMet-tRNA–AUG]
3. [IF-2–30S–fMet-tRNA–AUG] + 50S $\overset{GTP}{\longrightarrow}$ [70S–fMet-tRNA–AUG] + IF-2

GTP seems to be essential for Step **3**, but appears to have only a rate-enhancing effect on Step **2**. The recent observations of Fakunding *et al.** that radioactive IF-2 binds to 30S subunits, and that this binding is destabilized when a 70S initiation complex is formed in the presence of GTP, are consistent with Steps **1** and **3** above. A common mechanism (Steps **1** and **2**) is probably used by both prokaryotic and eukaryotic (2, 3) systems for polypeptide-chain initiation on the *small* subunit. However, the requirements for recycling of the corresponding initiation factors are different in the two systems, since the *Artemia salina* factor appears to function catalytically on the 40S subunit without GTP or the 60S subunits†.

The formation of the ternary complex elongation factor EF-T–GTP–aminoacyl-tRNA as an intermediate in polypeptide-chain elongation is well established (6), and the observations of Rudland *et al.* (7) have been interpreted as evidence for the formation of a similar IF-2–GTP–fMet-tRNA$_f$ complex in chain initiation (see also ref. 8). However, since, as shown in this paper, both the 30S binding and the proper positioning of fMet-tRNA can be accomplished without added GTP, it is unlikely that an IF-2–GTP–fMet-tRNA$_f$ complex is an obligatory intermediate in initiation.

Our current knowledge regarding the mechanism and requirements of prokaryotic polypeptide-chain initiation has been largely obtained from studies with purified initiation factors and ribosomal subunits previously exposed to high NH_4Cl and low Mg^{2+} concentrations. It remains to be seen whether native 30S subunits with their associated initiation

* Fakunding, J., Traugh, J. A., Traut, R. R. & Hershey, J. W. B. (1972) *Fed. Proc.* **31**, 410.

† Zasloff, M. & Ochoa, S., in preparation.

factors (IF-1, IF-2, and IF-3) show identical requirements for formation of the initiation complex.

I thank Dr. Herbert A. Klein and Mr. Michael Zasloff for their critical reading of the manuscript and Dr. Severo Ochoa for his interest in this work and for help in the preparation of the paper. Miss Jaroslava Husa provided excellent technical assistance. 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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