Competition between the Elongation Factors 1 and 2, and Phenylalanyl Transfer Ribonucleic Acid for the Ribosomal Binding Sites in a Polypeptide-synthesizing System from Brain*

(Received for publication, November 9, 1972)

Dietmar Richter†
From The Rockefeller University, New York, New York 10021

SUMMARY

80 S ribosomes and elongation factors 1 and 2 (EF-1 and EF-2), isolated from freeze-dried porcine brain of the hypothalamic region, were used to study the interrelation between the ribosomal binding of the two factors and of phenylalanyl-tRNA and N-acetyl-phenylalanyl-tRNA. Complexing of ribosomes with EF-2 + $\beta$, $\gamma$-methylene-guanosine 5'-triphosphate or EF-2 + GTP + fusidic acid prevented or inhibited EF-1-dependent binding of phenylalanyl-tRNA as well as EF-1-independent binding at 20 mM Mg$^{2+}$. On the other hand, when ribosomes were preincubated with EF-1, GTP, and phenylalanyl-tRNA, EF-2-dependent GDP binding became inhibited. Furthermore, ribosomes non-enzymatically charged with phenylalanyl-tRNA or N-acetyl-phenylalanyl-tRNA, presumably in the acceptor position, likewise did not accept EF-2 and GDP. These results indicate that EF-1 and EF-2 have an overlapping region on the 80 S ribosomes that includes the acceptor site for nonenzymatic binding of phenylalanyl-tRNA or N-acetyl-phenylalanyl-tRNA.

The peptide chain elongation process requires three bacterial elongation factors; two of them, EF-Tu and EF-G, function on ribosomes (1). Studies in several laboratories (2-5) have revealed, however, that the latter two cannot react simultaneously on 70 S ribosomes. This can be demonstrated under conditions where one elongation factor is fixed on the ribosomes while the complementary one is assayed for its function. Hence, E. coli ribosomes carrying EF-G no longer accept EF-Tu-directed Phe-tRNA binding and consequently EF-Tu-dependent GTP hydrolysis is also inhibited (2, 4, 5). These results suggest that both factors compete for a common region on the ribosomes and that this region is also involved in the hydrolysis of GTP.

The reverse, however, to prove whether ribosome-bound EF-Tu would inhibit the function of EF-G, turned out to be more complicated; the best result obtained was a 50% inhibition of the EF-G-dependent GTP binding to ribosomes carrying EF-Tu (2). One explanation for this less pronounced inhibition was thought to be the relatively high uncoupled GTPase activity of the bacterial EF-G (6). Since one is dealing with a rather heterogeneous ribosome population, it may be active in functioning with EF-G and GTP in an uncoupled reaction but inactive with EF-Tu, GTP, and Phe-tRNA.

In view of the low uncoupled activity (1) of EF-2, the eukaryotic equivalent of bacterial EF-G, it seemed desirable to explore an overlap between the two eukaryotic elongation factors EF-1 and EF-2; the former is equivalent to the bacterial EF-T. Previous results by Moldave et al. (7) had already shown that with each round of translocation the 80 S ribosomes and EF-2 undergo a cycle of association and dissociation suggesting, in light of the recent findings with the bacterial system, that EF-1 and EF-2 may react alternately with the 80 S ribosomes.

This report will deal with an eukaryotic system isolated from freeze-dried porcine brain of the hypothalamic region. The results indicate that EF-1 and EF-2 do share a common region on the 80 S ribosomes; in addition, Phe-tRNA as well as Ac-Phe-tRNA compete for this ribosomal binding site(s) for EF-1 and EF-2.

EXPERIMENTAL PROCEDURE

Materials

Freeze-dried hypothalamic fragments were obtained from Oscar Mayer, Inc., Madison, Wisconsin, and stored in liquid nitrogen. E. coli tRNA (Miles Laboratories) was charged with [14C]phenylalanine (specific activity 460 mCi per mmole) using the E. coli enzyme. Ac[3H]Phe-tRNA (specific activity of [3H]-phenylalanine, 7 Ci per mM) was prepared in a similar manner according to the method of Haseun and Chapeville (8). [3H]-GTP (9.2 Ci per mmole), [3H]GDP (9.5 Ci per mmole), and [14C]-GDP (specific activity 39.1 mCi per mmole) were from Schwarz-Mann. Gpp(CH$_3$)$_3$ was obtained from Miles. Fusidic acid was

* This work was supported by Grant GM-13972 to Fritz Lipmann from the United States Public Health Service. Some of these results were presented at the Cold Spring Harbor meeting on Protein Synthesis, September 5-8, 1972.

† Recipient of a fellowship from Deutsche Forschungsgemeinschaft. Present address, Max-Planck-Institut für Molekulare Genetik, Ihnestrasse 63-73, 1 Berlin 33 (Dahlem), Germany.

The abbreviation is: Gpp(CH$_3$)$_3$, $\beta$, $\gamma$-methylene-guanosine 5'-triphosphate.
a gift from Dr. W. Godtfredsen, Leo Pharmaceutical Products, Copenhagen, Denmark.

Methods

Preparation of 80 S Ribosomes, EF-1, and EF-2 from Porcine Brain of Hypothalamic Region

With this preparation, so far unsuccessful attempts have been made in this laboratory to prepare cell-free systems from the hypothalamus which might promote biosynthesis of small peptides produced by this tissue, such as the tripeptide, thyrotropin-releasing hormone. We became aware, however, of the high activity of this system in ribosome-poly(U)-dependent polyphenylalanine synthesis. Therefore, with this preparation the two elongation factors and 80 S ribosomes were isolated using a method described by Zomzely-Neurath (9). In 400 ml of Buffer A (50 mM Tris-HCl, pH 7.9, 25 mM KCl, 5 mM dithiothreitol, 5 mM MgCl₂, 0.25 mM sucrose) 20 g of freeze-dried hypothalamic fragments were suspended and kept at 4°C for 20 min. They were then homogenized in a glass tube with a Teflon pestle, and the homogenate was centrifuged at 15,000 × g for 10 min. The supernatant fraction was treated with deoxycholate, subjected to a discontinuous sucrose gradient, and centrifuged as described (9). The pellets were dissolved in small volumes of water and further purified by a salt-wash procedure. The ribosomes were incubated with puromycin at 0.5 mM KCl to release peptidyl-tRNA (10) and centrifuged through a 0.5 mM sucrose solution (9). The ribosomal pellets were kept in liquid nitrogen until used.

EF-1 and EF-2 were isolated from hypothalamus as described for other mammalian elongation factors (11-13), using Buffer A for homogenization of the fragments. The procedure included a Sephadex G-200 gel filtration step to separate EF-1 from EF-2 (11, 12) and chromatography of the elongation factors on hydroxyapatite (13). EF-1 was about 30% pure; however, further purification on a DEAE-cellulose column using a linear salt gradient (0.1 mM to 0.5 mM KCl in Buffer A) resulted in the loss of most of its activity. It is not clear whether this step caused a separation into subunits equivalent to the bacterial EF-Ts and EF-Tu. Recently, Moon et al. (14) reported on a subunit of the mammalian EF-1 that resembled the bacterial counterpart, EF-Tu. After DEAE-cellulose chromatography EF-2 was 65% pure (15). Optimal incorporation of phenylalanine was achieved with 40 to 50 μg of EF-1 and 10 to 40 μg of EF-2. Other conditions for optimal polyphenylalanine synthesis were as follows: 6 to 7 mM Mg²⁺, 150 mM KCl, and pH 7.8 to 8.0. The reaction was completed after 15 min at 37°C. The activity of the batches varied; under optimal conditions, 5 to 6 pmoles of phenylalanine were incorporated into hot trichloroacetic acid-insoluble material per min and per O.D. unit (A₂₆₀₉₉) of ribosomes. Similar results have been reported with other brain systems (16).

Enzymatic Binding of [³⁴C]Phe-tRNA to Ribosomes Charged with EF-2

First Incubation Step—Complex formation between ribosomes and EF-2 was carried out in the presence of unlabeled GTP. A total reaction mixture of 50 μl containing 20 mM Tris-HCl, pH 7.9, 50 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM GTP, 20 mM fusidic acid, 0.2 O.D. unit (A₂₆₀₉₉) of 80 S ribosomes, and 20 μg of poly(U), was preincubated with 10 to 40 μg of EF-2 at 4°C for 10 min. In some experiments, GTP and fusidic acid were replaced by 0.5 mM Gpp(3)P.

Second Incubation Step—In a separate experiment a reaction mixture of 20 μl containing 40 to 50 μg of EF-1, 0.1 mM GTP, 20 pmoles of [³⁴C]Phe-tRNA (specific activity 320 pmoles per mg of tRNA), 20 mM Tris-HCl, pH 7.9, 50 mM KCl, 5 mM MgCl₂, and 1 mM dithiothreitol was kept at 4°C for 10 min.

Third Incubation Step—After preincubation, both reaction mixtures were combined and incubated at 4°C for 2 min. The reaction mixture was diluted with 1 ml of Buffer B (20 mM Tris-HCl, pH 7.9, 50 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol) and passed through a Millipore filter. The latter was washed three times with 4 ml of Buffer B, dried, and counted in Liquifluor.

Nonenzymatic Binding of [³⁴C]Phe-tRNA or Ac[³¹H]Phe-tRNA to Ribosomes Charged with EF-2

Conditions were the same as for the enzymatic binding, except that the second incubation step was omitted. After the first step, 20 pmoles of [³⁴C]Phe-tRNA or 25 pmoles of Ac-Phe-tRNA were added; Mg²⁺ concentration was in the range from 2 mm to 30 mm. Incubation was carried out at 4°C for 2 min.

EF-2-directed Binding of [³¹H]GDP to Ribosomes Carrying Enzymatically Bound Phe-tRNA

First Incubation Step—The reaction mixture contained in a volume of 100 μl: 20 mM Tris-HCl, pH 7.9, 5 mM MgCl₂, 50 mM KCl, 1 mM dithiothreitol, 2.0 to 5.0 O.D. units (A₂₆₀₉₉) of 80 S ribosomes; 80 μg of poly(U), 100 μM GDP, 150 pmoles of [³¹H]GDP, and 100 μg of EF-1. The mixture was kept at 4°C for 20 min, diluted with 100 μl of Buffer B, and then filtered through a Sepharose 2B column (0.9 × 4.2 cm) using Buffer B. Fractions of 0.2 ml were collected and assayed for [³¹H]GDP radioactivity (10-μl aliquots). The ribosomal-bound [³¹H]Phe-tRNA that came off the column with the void volume was collected and used for the second incubation step. That these ribosomes carried not only [³¹H]Phe-tRNA but also EF-1 was indicated by adding the complementary factor EF-2; polyphenylalanine synthesis was obtained without newly added EF-1 (17, 18).

Second Incubation Step—A total volume of 100 μl contained 0.2 to 0.6 O.D. unit of ribosomes carrying 10 to 15 pmoles of [³¹H]GDP, and was incubated with 20 μg of EF-2, 60 pmoles of [³¹H]GDP, and 20 mM fusidic acid at 4°C for 5 min. The mixture was immediately passed through a Millipore filter; the latter was washed with 10 ml of Buffer C (20 mM Tris-HCl, pH 7.9, 5 mM MgCl₂, 1 mM dithiothreitol), dried, and counted in Liquifluor. A blank caused by EF-2 and [³¹H]GDP alone was subtracted.

EF-2-directed Binding of [³¹H]GDP or [³⁴C]GDP to Ribosomes Carrying Nonenzymatically Bound Phe-tRNA or Ac-Phe-tRNA

A total volume of 100 μl contained: 20 mM Tris-HCl, pH 7.9, 50 mM KCl, 20 μg of poly(U), 1 mM dithiothreitol, 1 to 2 O.D. units (A₂₆₀₉₉) of 80 S ribosomes, and 40 pmoles of Phe-tRNA or 30 pmoles of Ac-Phe-tRNA; Mg²⁺ concentration was in the range of 2 mm to 30 mm. The mixture was kept at 4°C for 30 min. Then 20 μg of EF-2 and 50 pmoles of [³¹H]GDP or 100 pmoles of [³⁴C]GDP were added; the fusidic acid concentration was 20 mM. After 1 min at 4°C, the reaction was stopped with 1 ml of Buffer C and passed through a Millipore filter as described above. The values were corrected by subtracting a blank caused by EF-2 and [³¹H]GDP or [³⁴C]GDP.

RESULTS

Influence of EF-2 on Binding to Ribosomes of EF-1 and [³⁴C]Phe-tRNA—First, 80 S ribosomes were charged with EF-2 and then they were subjected to EF-1-dependent [³⁴C]Phe-tRNA binding as outlined under “Methods.” Fig. 1 shows that EF-1-
FIG. 1. Enzymatic binding of [3H]Phe-tRNA to ribosomes precharged with GTP, fusidic acid, and various amounts of EF-2. Incubation steps were carried out as described under "Methods."

Fig. 2. Nonenzymatic binding of [3H]Phe-tRNA to ribosomes carrying EF-2 and Gpp(CH2)p at various Mg2+ concentrations. For details, see "Methods."

directed [3H]Phe-tRNA binding at 6 mM Mg2+ decreased with increasing concentrations of EF-2 prebound to the ribosomes in the presence of GTP and fusidic acid. However, even with rather high concentrations of EF-2 in the preincubation step, no complete inhibition of the enzymatic [3H]Phe-tRNA binding was observed. The upper curve in Fig. 1 indicates that in the absence of fusidic acid the ribosome-EF-2-GDP complex was rather labile and thus it was no longer possible to prevent EF-1-directed [3H]Phe-tRNA binding. Enzymatic Phe-tRNA binding was also inhibited by ribosomes precharged with EF-2 and Gpp(CH2)p (not shown). These experiments indicate that ribosomal-bound EF-2, like its bacterial counterpart, prevents EF-1 from binding Phe-tRNA to the acceptor site of the ribosomes.

---

TABLE I
Nonenzymatic binding of Ac[3H]Phe-tRNA to ribosomes precharged with EF-2

<table>
<thead>
<tr>
<th>Ribosomes preincubated</th>
<th>Nonenzymatic binding of Ac[3H]Phe-tRNA to ribosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alone</td>
<td>3.2</td>
</tr>
<tr>
<td>+ EF-2 + Gpp(CH2)p</td>
<td>0.7</td>
</tr>
<tr>
<td>+ EF-2 + GTP + fusidic acid</td>
<td>1.2</td>
</tr>
<tr>
<td>+ EF-2</td>
<td>3.4</td>
</tr>
</tbody>
</table>

---

TABLE II
EF-2-dependent binding of [3H]GDP to ribosomes precharged with EF-1 and Phe-tRNA

<table>
<thead>
<tr>
<th>Ribosomes preincubated</th>
<th>EF-2-dependent binding of [3H]GDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alone</td>
<td>5.9</td>
</tr>
<tr>
<td>EF-1, GTP, Phe-tRNA</td>
<td>1.9</td>
</tr>
<tr>
<td>EF-1, Phe-tRNA</td>
<td>5.2</td>
</tr>
<tr>
<td>EF-1</td>
<td>4.7</td>
</tr>
<tr>
<td>EF-1</td>
<td>6.1</td>
</tr>
</tbody>
</table>

* For details, see "Methods."

Influence of EF-2 on Nonenzymatic Binding to Ribosomes of [3H]Phe-tRNA or Ac[3H]Phe-tRNA—As shown in Fig. 2, ribosomes carrying EF-2 and Gpp(CH2)p no longer accepted [3H]Phe-tRNA at high Mg2+ concentrations, whereas when EF-2 was omitted [3H]Phe-tRNA bound very well (upper curve of Fig. 2). Ribosomes complexed in the presence of EF-2, GTP, and fusidic acid were also inactive in Phe-tRNA binding at 20 mM Mg2+ (not shown). A similar result was obtained when studying the non-enzymatic binding of Ac[3H]Phe-tRNA (Table I). Ribosomes were preincubated as outlined in the left column of the table and were then incubated with Ac[3H]Phe-tRNA at 20 mM Mg2+. Lines 2 and 3 indicate that Ac[3H]Phe-tRNA binding was blocked with ribosomes precharged with EF-2 and Gpp(CH2)p or EF-2, GTP, and fusidic acid. Recent reports have shown (19, 20), that in contrast to the bacterial system, peptidyl-tRNA or analogs such as Ac-Phe-tRNA preferentially enter the acceptor position on 80 S ribosomes. Hence, Ac[3H]Phe-tRNA binding, like Phe-tRNA binding, was inhibited by ribosomal-bound EF-2.

Influence of Ribosome-bound Aminoacyl-tRNA on Binding of EF-2—Enzymatic binding of Phe-tRNA to ribosomes was carried out as outlined under "Methods." Ribosomes reisolated by gel filtration on Sepharose columns carried Phe-tRNA as well as EF-1 (17). As indicated in Table II, these ribosomes were no longer active in complex formation with EF-2, [3H]GDP, and fusidic acid (Line 2), indicating that EF-2 could not enter into its ribosomal-binding region. A slight inhibition of the EF-2 function was also observed with ribosomes preincubated only with EF-1 and GTP (Line 4), suggesting that EF-1 binds rather easily to its binding region on 80 S ribosomes even in the absence of Phe-tRNA. No inhibition occurred when GTP was omitted from the preincubation step (Lines 3 and 5).

It thus appears that once bound to the ribosomes, either one of the two factors blocks the function of its counterpart. These
Phe-tRNA was bound to the ribosomes and EF-2-dependent when the Mg2+ concentration was as low as 6 mM Mg2+, no Ac-Phe-tRNA was absent, no Phe-tRNA binding took place and hence not inhibited (upper curve). An experiment with ribosomes carrying prebound Ac-Phe-tRNA at 20 mM Mg2+ also resulted in the inhibition of EF-2-dependent [3H]GDP binding. An experiment with ribosomes carrying nonenzymatically prebound Ac-Phe-tRNA at 20 mM Mg2+ and 20 mM Mg2+ in the absence of EF-1.

Mammalian EF-1 and EF-2 compete for a common region on 80 S ribosomes. EF-1-directed [3H]Phe-tRNA binding was prevented when ribosomes were precharged with EF-2 and GTP, or with EF-2, GTP, and fusidic acid. Similar competition was observed with the reverse reaction; EF-2-dependent [3H]GDP binding to ribosomes did not take place when the latter carried EF-1 and Phe-tRNA. Although both elongation factors seem to share a common region on the ribosomes, their activity has not yet been related to specific proteins, whereas two acidic proteins (21-24) that were isolated from 80 S ribosomes of E. coli were shown to be connected with the functions of EF-G and EF-Tu (25-27).

Furthermore, the data reported here indicate that in addition to the mutual exclusion of EF-1 and EF-2 in binding to 80 S ribosomes, the ribosomal acceptor positions of Phe-tRNA and Ac-Phe-tRNA also seem to be affected by the two factors and vice versa. Nonenzymatic binding of [3H]Phe-tRNA or of [3H]Ac-Phe-tRNA was inhibited by ribosomes precharged with EF-2 and Gpp(CH2)p or EF-2, GTP, and fusidic acid. In the reverse experiment, ribosomes carrying nonenzymatically bound Phe-tRNA or Ac-Phe-tRNA in the acceptor position did not form a complex with EF-2, [3H]GDP, and fusidic acid; a similar observation has been made for other systems (28, 29).

Our results may reflect either identical binding sites for both EF 2 and aminoacyl tRNA, or these sites are separate but located so close together that under certain conditions they interfere with each other. The latter explanation seems more likely, since to fulfill its function as translocase, EF-2 has to interact with ribosomes carrying Phe-tRNA or Ac-Phe-tRNA in the acceptor position; on the other hand, the finding that the function of EF-2 is blocked by nonenzymatically bound Phe-tRNA or Ac-Phe-tRNA conflicts with this idea. However, one should consider that all of the experiments reported here and elsewhere (2-5) on the competition of the two factors for a ribosomal binding site were carried out by measuring in an indirect way elongation factor-dependent functions rather than in a direct way by binding radioactively labeled elongation factors to the ribosomes. In the experiment in which the function of EF-2 was studied, the indicator was either [3H]GDP or [3H]GTP. Hence it cannot be completely excluded that ribosomes nonenzymatically precharged with Phe-tRNA or Ac-Phe-tRNA may accept EF-2 but not the guanosine nucleotide. Furthermore, the experimental conditions described here permit the study of single steps of the peptide chain elongation process rather than following the overall reaction. (a) Gpp(CH2)p can replace GTP in ribosomal binding of aminoacyl-tRNA and it can also replace GTP in the formation of the ribosome-EF-2-GDP complex, yet the overall synthesis of a polypeptide depends completely on GTP. (b) Fusidic acid causes the “freezing” of the ribosome-EF-2-GDP complex, but this results in the inhibition of the translational step. (c) Although formation of the ribosome-EF-2-GDP complex as well as the ribosomal binding of aminoacyl-tRNA takes place at 4°C, the translational step requires higher temperatures. The latter aspect is of interest since preliminary experiments in this laboratory have indicated that at the elevated temperature the complex formation between EF-2, GDP, and fusidic acid, and ribosomes nonenzymatically precharged with Phe-tRNA, is less inhibited. Similar results have been observed by Modolell and
Vazquez (28). Previously, Skogerson and Moldave (30) using more physiological conditions showed that ribosomes carrying endogenous peptidyl-tRNA could bind EF-2.

That the interaction of the elongation factors with their ribosomal binding region is complex is indicated in a report by Beres and Lucas-Lenard (31) who have recently shown that EF-G from Pseudomonas fluorescens bound to E. coli ribosomes does not prevent EF-Tu-directed Phe-tRNA binding, whereas the homologous E. coli EF-G factor does; as is known, both factors are completely interchangeable in the overall reaction as well as in the partial reactions of peptide chain elongation (1).

Acknowledgment—The author gratefully acknowledges the continued interest and encouragement of Dr. Fritz Lipmann as well as the helpful discussions during the preparation of this manuscript.

REFERENCES
Competition between the Elongation Factors 1 and 2, and Phenylalanyl Transfer Ribonucleic Acid for the Ribosomal Binding Sites in a Polypeptide-synthesizing System from Brain

Dietmar Richter


Access the most updated version of this article at http://www.jbc.org/content/248/8/2853

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/248/8/2853.full.html#ref-list-1