THE BINDING OF THE PYROPHOSPHORYL TRANSFERASE AND THE ELONGATION FACTOR Tu AND G TO RIBOSOMES FROM *ESCHERICHIA COLI*

Ursula KLEINERT* and Dietmar RICHTER**

**Max-Planck-Institut für Molekulare Genetik, Abt. Wittmann, Berlin-Dahlem and Institut für Physiologische Chemie, Abt. Zellbiochemie, Universität Hamburg, West Germany*

Received 10 March 1975
Revised version received 14 May 1975

1. Introduction

It has been shown that the peptide chain elongation factor Tu (EF-Tu) and G (EF-G) from bacteria interact on ribosomes at a common region (for reviews see [1,2]). The existence of such a region has been demonstrated by competition experiments where ribosomes carrying EF-G are inactive in the EF-Tu-dependent Phe-tRNA binding reaction. Vice versa ribosomes precharged with EF-Tu do not function in EF-G directed reactions such as GTP hydrolysis or GDP binding. Based on these results it has been concluded that the two peptide factors react alternately on ribosomes and that both not only recognize but also bind to identical site(s) on the ribosome, although conclusive evidence for the latter point has not yet been obtained.

Recently another protein, the stringent factor, has been found to function on *Escherichia coli* robosomes [3]. The protein factor catalyzes the production of guanosine-5′-diphosphate-3′-diphosphate (ppGpp) and guanosine-5′-triphosphate-3′-diphosphate (pppGpp). In this reaction a pyrophosphate group is transferred from ATP to GDP or GTP provided uncharged and codon-specific tRNA is bound to the acceptor site of the ribosome [4,5]. The protein factor, the pyrophosphoryl transferase (stringent factor), is associated with 70S ribosomes and 50S but not with 30S subunits [6,7]. It is not known whether the pyrophosphoryl transferase and the elongation factors bind to and react alternately with identical site(s) on the ribosome.

The present data are concerned with the direct binding of the two elongation factors and the pyrophosphoryl transferase to ribosomes. We will show that [^{3}H] EF-G and [^{3}H] EF-Tu interact with identical ribosomal region(s) whereas the pyrophosphoryl transferase binds to a site different from that of EF-Tu or EF-G.

2. Materials and methods

2.1. *Materials*

E. coli strain CGSC 2834a was grown in a tryptone-glucose-yeast extract medium [8]. [^{14}C] phenylalanine (spec.act. 460 mCi/mmol), [^{3}H] GDP (spec.act. 1.29 Ci/nmol) and α-[^{32}P] GTP (spec.act. 25 Ci/mmol) were obtained from New England Nuclear Corp., Boston; sodium [^{3}H] borohydride (specific activity 6-40 Ci/mmol) came from Amersham and Buchler, Braunschweig, Germany. β,γ-methylene guanosine-5′-triphosphate (GMPPCP) was purchased from Miles Laboratories. *N*-2-Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) came from Serva, Heidelberg. EF-Tu [9], EF-G [9] and the pyrophosphoryl transferase [8,16] from *E. coli* were prepared as described; 70S ribosomes (1 A_{260} unit ≙ 25 pmol), 50S (1 A_{260} unit ≙ 39 pmol) and 30S (1 A_{260} unit ≙ 67 pmol) ribosomal subunits were isolated by zonal centrifugation [8]. Assays

** To whom reprint requests should be sent

North-Holland Publishing Company – Amsterdam

for [^{3}H] GDP binding to EF-Tu [9], enzymatic binding of [^{14}C] Phe-tRNA (spec.act. 400 pmol [^{14}C] phenylalanine/mg tRNA) to ribosomes [10], complex formation of EF-G, GDP, fusidic acid and ribosomes [10], polyphenylalanine synthesis [11] and pyrophosphoryl transferase activity [3] were carried out as reported.

2.2. *Radioactive labeling of the protein factors*

Tritiated pyrophosphoryl transferase or elongation factors were prepared by the method of Means and Feeney [12] except that borate was replaced by HEPES buffer.

9.6 mg/ml elongation factor Tu were dialysed against 2 X 2-liter buffer 1 (10 mM HEPES, 10 mM Mg-acetate, 10 μM GDP; the pH was adjusted to 9.0 with NaOH). Reductive methylation was carried out in a total vol of 1.62 ml containing 7.7 mM HEPES, pH 9.0, 7.7 mM Mg-acetate, 7.7 μM GDP, 600-700 μg EF-Tu. The reaction was started with the addition of formaldehyde (final concentration was 3 mM or as indicated in the legend to fig.1), then four times 5 μl sodium [^{3}H] borohydride (4.5 mg/ml; 220–1000 mCi/mg) were added in intervals of 30 sec; the mixture was kept at 4°C for 10 min and was then dialysed against 3 X 1-liter buffer 1 and 5 X 1-liter buffer 2 (10 mM Tris–HCl, pH 7.5, 10 mM Mg-acetate, 10 μM GDP). In a similar way the tritiated EF-G or pyrophosphoryl transferase was prepared except that GDP was omitted. The tritiated factors were stored in liquid nitrogen in small samples until used. For calculation of the radioactivity an aliquot of the labeled factor was precipitated with 10% trichloroacetic acid, collected on a Whatman glass fiber filter (No.6, ϕ 23 mm) and counted in

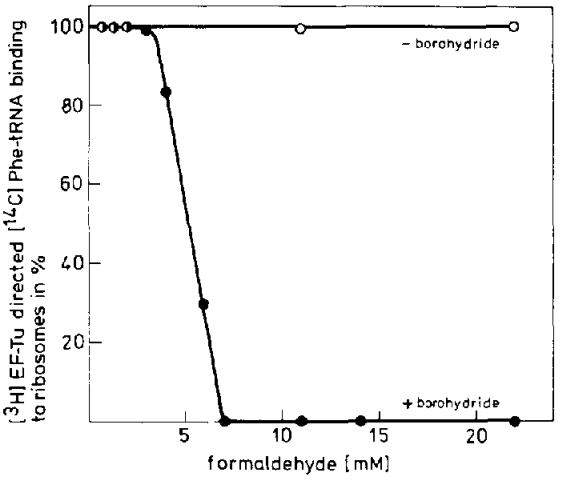

Fig.1. Reductive methylation of EF-Tu in the presence of various concentrations of formaldehyde. 672 μg EF-Tu were incubated in the presence of various concentrations of formaldehyde with or without [^{3}H] borohydride as described above. [^{3}H] EF-Tu was assayed for ribosomal [^{14}C] Phe-tRNA binding activity [10].

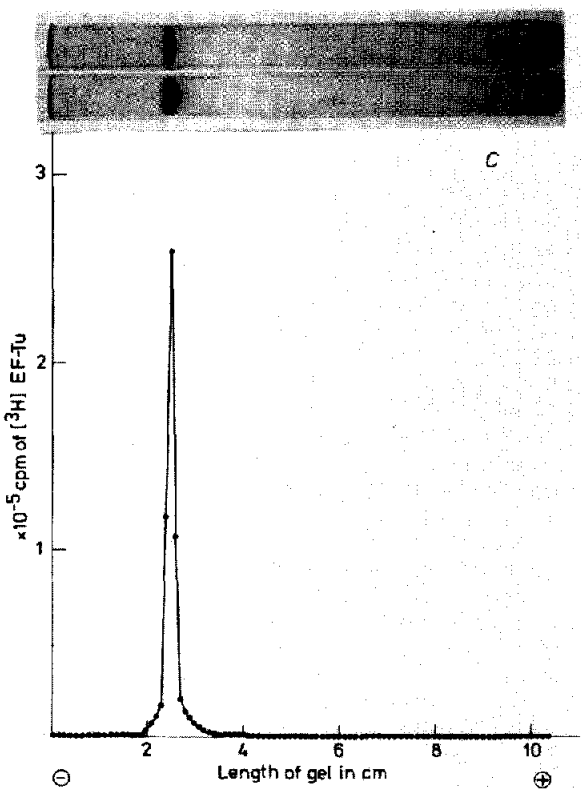

Fig.2. Comparison of the electrophoretic properties of labeled and unlabeled EF-Tu. SDS-polyacrylamide gel electrophoresis (10% acrylamide) was carried out according to Weber and Osborn [14]. Gel A was run with 12 μg EF-Tu, gel B and C were run with 12 μg [^{3}H] EF-Tu. Current was 13 mA/tube for 3.5 hr. Gel A and B were fixed for 30 min in 50% TCA and then stained for 30 min in a solution consisting of 0.1%. Coomassie brillant blue and 50% TCA [15]. Destaining was carried out in 7% acetic acid. Gel C was cut in 1 mm slices. Each slice was treated with 200 μl SDS (concentration 1%) for 12 hr at 37°C. After centrifugation aliquots of the supernatant fractions were measured for radioactivity in Bray's solution.

Table 1
Functional properties of labeled and unlabeled pyrophosphoryl transferase and elongation factors

Protein factors	Synthesis of Polyphenylalanine (pmoles)	Synthesis of pppGpp + ppGpp (percent)
[^{3}H] EF-Tu + [^{3}H] EF-G	2.5	
[^{3}H] EF-Tu + EF-G	2.4	
EF-Tu + [^{3}H] EF-G	2.6	
EF-Tu + EF-G	2.7	
[^{3}H] Pyrophosphoryl transferase		67.8
Pyrophosphoryl transferase		75.1

Polyphenylalanine synthesis was measured by the method of Conway and Lipmann [11]; production of pppGpp + ppGpp was carried out as reported by Haseltine et al. [3].

Bray's solution. The protein concentration was estimated by the method of Lowry et al. [13].

As depicted in fig.1 tritiated EF-Tu survives conditions for reductive methylation provided the formaldehyde concentrations did not exceed 3 to 4 mM; under these conditions the specific activity of the tritiated factor was 1–10 Ci/mmol depending on the specific activity of the commercial available sodium [^{3}H] borohydride. Similar results were obtained for EF-G and the pyrophosphoryl transferase. The reductive methylation method neither altered the electrophoretic (fig.2) nor affected the functional properties (table 1) of the protein factors.

3. Results and discussion

One reason for preparing radioactively labeled factors was to study the binding of the various factors to the ribosome. As outlined in the legend to fig.3 [^{3}H] EF-Tu was incubated with ribosomes,

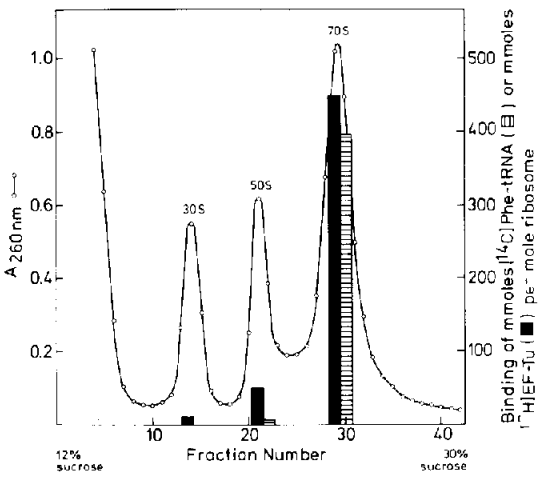

Fig.3. Binding of [^{3}H] EF-Tu to ribosomes. 50S subunits (1200 pmol/ml) and 30S subunits (1200 pmol/ml) were first preincubated with 200 μg/ml of poly U, 40 mM Tris–HCl, pH 7.8, 10 mM Mg-acetate and 4 mM dithiothreitol; the reaction volume was 250 μl. Preincubation was carried out at 30°C for 20 min; the mixture was cooled to 4°C (reaction mixture I). Reaction mixture II (250 μl) was incubated with 40 mM Tris–HCl, pH 7.8, 4 mM dithiothreitol, 100 mM NH_4Cl, 1 mM GMPPCP, 2000 pmol/ml of [^{3}H] EF-Tu ([^{3}H] EF-Tu was diluted with cold EF-Tu to yield a specific activity of 0.4 Ci/mmol; mol. wt of EF-Tu: 45 000) and 800 pmol/ml of [^{14}C] Phe-tRNA at 4°C for 10 min. Reaction mixtures I and II were combined (final Mg concentration was 5 mM), kept at 30°C for 10 min, then applied to a 12 to 30% sucrose gradient (w/v; in 20 mM Tris–HCl buffer, pH 7.8, 2 mM dithiothreitol, 40 mM NH_4Cl, 15 mM Mg-acetate) and centrifuged in a SW 27 rotor at 20 000 rev/min for 14 hr. The gradient was analyzed in an Isco gradient fractionator; 46 fractions with 0.8 ml per tube were collected; 200 μl aliquots of the fractions were counted in Bray's solution. In a control experiment where GMPPCP and [^{14}C] Phe-tRNA had been omitted less than 40 mmol of [^{3}H] EF-Tu per mol of 70S ribosomes were bound (not shown).

GMPPCP, and [^{14}C] Phe-tRNA, and applied to a sucrose density gradient. 400 mmol of [^{14}C] Phe-tRNA and 450 mmol of [^{3}H] EF-Tu were bound per mol 70S ribosomes (fig.3). Thus about 40% of the formed 70S ribosomes were active in binding EF-Tu and Phe-tRNA. When ribosomes were complemented with [^{3}H] EF-G, GDP, and fusidic acid [10], 350 mmol of tritiated EF-G were bound per mol ribosome (data not shown).

To see whether ribosomal bound, but unlabeled EF-G directly interfered with the binding of tritiated EF-Tu, ribosomes were complexed with EF-G, fusidic acid and GDP and isolated by centrifugation [10]; the complexed ribosomes were analysed for [^{3}H] EF-Tu and [^{14}C] Phe-tRNA binding. Table 2 demonstrates that ribosomal binding of [^{3}H] EF-Tu and [^{14}C] Phe-tRNA is significantly reduced when ribosomes were precharged with EF-G, GDP, and fusidic acid; without GDP and/or fusidic acid the ribosomal binding of the radioactive material was not reduced which is in line with previous results that GDP and fusidic acid are required for the formation of a stabile ribosome–EF-G complex. Our experiments directly show that EF-G bound to ribosomes not only inhibits the function of EF-Tu but also blocks the binding of this factor. This finding is in agreement with previous experiments showing the mutual exclusion of the two elongation factors [1,2].

In order to study the relationship between ribosomal regions involved in stringent and elongation factor functions attempts have been made to find optimal conditions for binding the pyrophosphoryl transferase to the ribosome. In principle ribosomes incubated with tritiated pyrophosphoryl transferase under various conditions were isolated by centrifugation and assayed for guanosine polyphosphate production and for ribosomal bound [^{3}H] pyro-

Table 2
Binding of [^{3}H] EF-Tu to ribosomes precharged with unlabeled EF-G, fusidic acid and GDP

Ribosomes preincubated	Binding of [^{3}H] EF-Tu (pmoles/pmole of ribosomes)	Binding of [^{14}C] Phe-tRNA (pmoles/pmole of ribosomes)
Alone	0.35	0.31
+ EF-G	0.29	0.30
+ EF-G + fusidic acid	0.34	0.35
+ EF-G + GDP	0.34	0.32
+ EF-G + fusidic acid	0.07	0.06

Ribosomes carrying EF-G were isolated as reported [10]: Ribosomes were preincubated as outlined in the left column, centrifuged and dissolved in buffer containing 20 mM Tris–HCl, pH 7.8, 2 mM dithiothreitol, 5 mM Mg-acetate. [^{3}H] EF-Tu and [^{14}C] Phe-tRNA binding to these ribosomes were carried out in a total vol of 250 μl; the reaction mixture contained 1500 pmol/ml ribosomes charged with or without EF-G (in a parallel experiment it was determined that 1 pmole of ribosomes carried 0.25 to 0.35 pmol of [^{3}H] EF-G when complexed with GDP and fusidic acid [10]), 160 μg/ml of poly U, 20 mM Tris–HCl, pH 7.8, 10 mM Mg-acetate, 4 mM dithiothreitol. Incubation was at 30°C for 10 min (reaction mixture I). Reaction mixture II and incubation conditions were the same as described in the legend to fig.3. Reaction mixtures I and II were combined, incubated and layered on top of 1.6 ml of a 5% sucrose solution (w/v; in buffer 3: 20 mM Tris–HCl buffer, pH 7.8, 15 mM Mg-acetate, 2 mM dithiothreitol). Centrifugation was performed at 150 000 *g* for 3.5 hrs in a Beckman 50-Ti rotor. The ribosomal pellet was carefully rinsed with 3 × 2 ml buffer 3 and finally dissolved in 50 μl of the same buffer. Aliquots were analyzed for radioactivity and for absorbance at 260 and 280 nm.

Table 3
Binding of tritiated pyrophosphoryl transferase to 70S ribosomes

Incubation of the [^{3}H] pyrophosphoryl transferase with	Ribosomal binding of [^{3}H]pyrophosphoryl transferase (cpm)
30S	280
50S	510
30S + 50S	2110
30S + 50S + poly U	6350
70S-poly U	7300
70S-poly U + $tRNA^{Phe}$	7560
70S-poly U + ATP	7130
70S-poly U + GTP	7450

The 70S-poly U complex was prepared as outlined in the legend to fig.3; in the experiment described here this mixture is referred to as mixture I. Where indicated 30S or 50S and/or poly U were omitted from mixture I. Binding of the [^{3}H] pyrophosphoryl transferase was carried out in a total volume of 500 μl: 250 μl of mixture I were mixed with 250 μl mixture II containing the labeled transferase and where indicated $tRNA^{Phe}$, ATP or GTP; the final concentrations were 20 mM Tris· HCl, pH 7.8, 15 mM Mg-acetate, 2 mM dithiothreitol, 10 μg [^{3}H] pyrophosphoryl transferase (45 000 cpm/μg protein), 0.4 mM ATP or GTP, 25 μg $tRNA^{Phe}$. The reaction mixture was kept at 4°C for 30 min and passed through a 5% sucrose solution as outlined in the legend to table 2. The ribosomal pellet was dissolved in 50 μl buffer 3, and 5 μl aliquots were counted in Bray's solution.

phosphoryl transferase. As shown in table 3 the pyrophosphoryl transferase preferentially co-sedimented with the 70S–mRNA complex but not with the 50S or 30S ribosomal subunit. Omission of poly U reduced the ribosomal binding of the pyrophosphoryl transferase. Uncharged tRNA or ATP or GTP added prior the centrifugation step did not enhance the formation of the 70S mRNA-pyrophosphoryl transferase complex. Optimal binding of the tritiated pyrophosphoryl transferase to the ribosome occured at 5 to 10 mM Mg-acetate.

In order to find out whether the pyrophosphoryl transferase and the elongation factors bind to identical or overlapping region(s) on the ribosome, the latter complexed with the pyrophosphoryl transferase were analyzed for [^{3}H] EF-Tu or [^{3}H] EF-G binding. Table 4 shows that neither [^{3}H] EF-Tu nor [^{3}H] EF-G binding was prevented by the ribosomal bound transferase.

Table 4
Binding of the pyrophosphoryl transferase and [^{3}H] EF-Tu or [^{3}H] EF-G to ribosomes

Conditions	Binding of [^{3}H] EF-Tu (pmoles/pmole of 70S ribosomes)	Binding of [^{3}H] EF-G (pmoles/pmole of 70S ribosomes)
70S ribosomes	0.41	0.35
70S-pyrophosphoryl transferase complex	0.39	0.37

Formation and isolation of the 70S–poly U-pyrophosphoryl transferase complex was carried out as outlined in the legend to table 3 except that unlabeled pyrophosphoryl transferase was used. Binding of the tritiated elongation factors to the ribosome-pyrophosphoryl transferase complex was carried out as described in the legend to fig.3.

To exclude the possibility that the pyrophosphoryl transferase was released from the ribosomes when incubated with the elongation factor, the 70S–poly U complex charged with [^{3}H] pyrophosphoryl transferase was isolated by centrifugation and incubated with Phe-tRNA, GMPPCP, and unlabeled EF-Tu. After incubation at 30°C for 10 min the ribosomes were re-isolated by centrifugation and analyzed for ^{3}H-radioactivity. Table 5 shows that there is no significant release of ^{3}H-pyrophosphoryl transferase from the ribosomes when incubated with EF-Tu. We interprete this result that pyrophosphoryl transferase and elongation factor Tu can react simultanoulsy on the ribosomes.

Our data indicate that the *E. coli* pyrophosphoryl transferase binds to a site different from that of the elongation factors. These findings are in good agreement with previous experiments in which it was shown that the two acidic proteins L7/L12 from the large ribosomal subunit are not required for the pyrophosphoryl transfer reaction [17,18], whereas they are for EF-Tu and EF-G functions [1,2]. The possibility that transferase and elongation factors do not bind to the same but rather to different ribosomes is unlikely, because ribosomes precharged with EF-Tu or EF-G were inactive in the pyrophosphoryl transferase-dependent formation of pppGpp and ppGpp but active in binding the transferase [16]. The inhibition of the synthesis of guanosine

Table 5
Incubation of the ribosome [^{3}H]pyrophosphoryl transferase complex with EF-Tu, GMPPCP, and Phe-tRNA

Incubation conditions	Ribosomal bound [^{3}H]pyrophosphoryl transferase (cpm)
Alone	3650
+ EF-Tu + Phe-tRNA + GMPPCP	3250
+ EF-Tu + Phe-tRNA	3420
+ EF-Tu	3180

Binding of the [^{3}H]pyrophosphoryl transferase to the 70S-poly U complex and isolation of the complexed ribosomes were carried out as outlined in the legend to table 3. The ribosomal pellet was dissolved in buffer 3 (50 μl) and complemented with EF-Tu, Phe-tRNA, and GMPPCP as shown in the left column of the Table. The total reaction vol was 500 μl and the conditions for binding EF-Tu to the ribosomes were the same as described in the legend to fig.3. After incubation the 500 μl reaction mixture was layered on top of a 5% sucrose solution and centrifuged as described (see table 2). The ribosomal pellet was dissolved in buffer 3 (50 μl) and 10 μl aliquots were counted in Bray's solution.

polyphosphates was due to the blocking of the acceptor site of the peptidyltransferase center by the elongation factor and/or aminoacyl-tRNA [16].

Acknowledgements

We would like to thank Drs. H. G. Wittmann and R. R. Crichton for helpful discussions.

References

[1] Haselkorn, R. and Rothman-Denes, L. B. (1973) Ann. Rev. Biochem. 42, 397–438.
[2] Pongs, O., Nierhaus, K. H., Erdmann, V. A. and Wittmann, H. G. (1974) FEBS Lett. 40, 28–37.
[3] Haseltine, W. A., Block, R., Gilbert, W. and Weber, K. (1972) Nature 238, 381–383.
[4] Pedersen, F. S., Lund, E. and Kjeldgaard, N. O. (1973) Nature 243, 13–15.
[5] Haseltine, W. A. and Block, R. (1973) Proc. Natl. Acad. Sci. USA 70, 1564–1568.
[6] Ramagopal, S. and Davis, B. (1974) Proc. Natl. Acad. Sci. USA 71, 820–824.
[7] Richter, D. and Isono, K. (1974) FEBS Lett. 44, 270–273.
[8] Richter, D., Erdmann, V. A. and Sprinzl, M. (1974) Proc. Natl. Acad. Sci. USA 71, 3226–3229.
[9] Arai, K., Kawakita, M. and Kaziro, Y. (1972) J. Biol. Chem. 247, 7029–7037.
[10] Richter, D. (1972) Biochem. Biophys. Res. Commun. 46, 1850–1856.
[11] Conway, T. W. and Lipmann, F. (1964) Proc. Natl. Acad. Sci. USA 52, 1462–1469.
[12] Means, G. E. and Feeney, R. E. (1968) Biochemistry 7, 2192–2201.
[13] Lowry, O. H., Rosebrough, W. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[14] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
[15] O'Farell, P., Gold, L. and Huang, W. H. (1973) J. Biol. Chem. 248, 5499–5501.
[16] Richter, D., Nowak, P. and Kleinert, U. (1975) submitted for publication.
[17] Richter, S. (1973) FEBS Lett. 34, 291–294.
[18] Lund, E., Pedersen, S. and Kjeldgaard, N. O. (1973) in: 1st Symposium on Ribosomes and RNA Metabolism, Slovak Academy of Sciences, Bratislava, Czechoslovakia, p. 367.