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# The Binding of Tritiated Elongation Factors 1 and 2 to Ribosomes from Krebs II Mouse Ascites Tumor Cells

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Tritiated elongation factors 1 and 2 (EF-1 and EF-2) were obtained from Krebs II ascites cells which had been grown in mice injected with radioactive amino acids. The highly purified factors were sufficiently radioactive to be used in a study of the interactions between ribosomes and elongation factors.

The following results were obtained.

1. EF-1 binding to ribosomes requires the presence of a polynucleotide, an aminoacyl-tRNA specified by the latter and a guanosine nucleotide carrying three phosphate groups. The hydrolysis of the GTP molecule involved in the binding reaction leads to the immediate release of EF-1. If GTP is replaced by Guo-5′-$P_2$-$CH_2$-$P$ the factor remains bound to the ribosome and can be detected by sucrose gradient centrifugation techniques.

2. Likewise EF-2 binding to ribosomes can only be detected in the presence of Guo-5′-$P_2$-$CH_2$-$P$.

3. The affinity of ribosomes for EF-2 appears to be higher than for EF-1: preincubation of ribosomes with EF-2 inhibits the subsequent attachment of EF-1 almost completely. EF-1 prebound to ribosomes in the presence of Guo-5′-$P_2$-$CH_2$-$P$, poly(uridylic acid) and Phe-tRNA$^{Phe}$ is partially removed from the ribosomes together with Phe-tRNA during a second incubation with EF-2.

4. Although EF-2 binding to ribosomes precludes any stable association between ribosomes and EF-1 it does not prevent the insertion of aminoacyl-tRNA into the ribosomal A-site. The attachment of aminoacyl-tRNA under these conditions enhances the binding of EF-2 to the ribosome.

5. The antibiotic showdomycin strongly inhibits the attachment of EF-1 to ribosomes and to a lesser degree impairs the binding of EF-2.

6. A-site ribosomes display a strong preference for the attachment of EF-2 and bind EF-1 only very poorly. The reverse is true for P-site ribosomes which are good substrates for the binding of EF-1 and bind EF-2 less efficiently than A-site ribosomes.

These results and a number of additional findings made in this and in previous studies are discussed in the general context of the structure and function of mammalian elongation factors 1 and 2.

Elongation factor 1 (EF-1) from ascites tumor cells occurs in multiple forms. All of these appear to be composed of aggregates of different numbers of a single polypeptide chain with an approximate molecular weight of 47000 [1]. The pure tetramer form of EF-1 was shown to carry one binding site for guanosine nucleotides which can be occupied by GTP, GDP or Guo-5′-$P_2$-$CH_2$-$P$ [2]. The binding constants of EF-1 for both GDP and GTP appear to be approximately $10^5$ $M^{-1}$ but only the binding of GTP conveyed to elongation factor 1 the capacity to specifically interact with the aminoacyl-tRNA [2]. The fact that Guo-5′-$CH_2$-$P$ could promote the EF-1-directed binding of aminoacyl-tRNA to ribosomes [3], while only promoting slight aminoacyl-tRNA protection against deacylation in an EF-1-dependent protection assay [2], indicated that these two functions of the factor may not be comparable.

In view of these observations, studies based on direct measurements of the interactions of [$^3$H]EF-1 and [$^3$H]EF-2 with the ribosome seemed necessary. Earlier studies on the interaction of elongation factors and ribosomes involved the use of antibiotics [4,5],

*Abbreviations.* EF-1, elongation factor 1; EF-2, elongation factor 2; Guo-5′-$P_2$-$CH_2$-$P$, 5′-guanylyl-methylene-diphosphonate.

the binding of labelled aminoacyl-tRNA [6,7] and the binding of labelled nucleotides [8–10]. However, in order to obtain direct and clear-cut data in this area, it appeared essential to prepare mammalian elongation factors of high chemical purity, biological activity and adequate specific radioactivity.

Using factors which met these requirements we now present evidence to answer some of the questions which have been of interest in recent years. Amongst these are the following.

a) Does EF-1 remain bound to the ribosome, after attaching aminoacyl-tRNA?

b) Does EF-2 remain on the ribosome after translocation occurs?

c) Do direct studies of this type support the suggestions that EF-1 and EF-2 binding are mutually exclusive?

d) Can ribosomes with peptidyl-tRNA in the A-site or P-site be shown to react with the two factors in different ways?

e) Is it possible to demonstrate specific interactions between ribosomal subunits and $^3$H-labelled elongation factors?

## EXPERIMENTAL PROCEDURE

### *Chemicals*

[$^3$H]Phenylalanine (specific activity 5 Ci/mmol) and [$^{14}$C]phenylalanine (specific activity 400 mCi/mmol) as well as a number of tritiated amino acids were obtained from the Radiochemical Centre (Amersham, England). Poly(uridylic acid), creatine phosphate and creatine kinase were purchased from Boehringer Mannheim GmbH (Mannheim, Germany). Guanosine triphosphate (GTP), guanosine diphosphate (GDP) and glutathione came from Serva Laboratories (Heidelberg, Germany). 5′-Guanylylmethylene-diphosphonate (Guo-5′-$P_2$-$CH_2$-$P$) was a product of Miles Laboratories (Kankakee, Illinois, U.S.A.). All other chemicals came from Merck KG (Darmstadt, Germany) and were of the highest purity available. DEAE-cellulose (Whatman DE 52) and cellulose phosphate (Whatman P-11) were from W.-R. Balston Ltd (Maidstone, Kent, England).

### *Labelling of Elongation Factors* in vivo

On the third day after being injected with 0.2 ml of Krebs II ascites fluid, mice were given two intraperitoneal injections of 100 μCi of an approximately equimolar solution of L-[$^3$H]alanine, L-[$^3$H]valine, L-[$^3$H]tyrosine, L-[$^3$H]lysine and L-[$^3$H]leucine, each of specific radioactivity greater than 20 Ci/mmol. The second injection was given 8 h after the first. The same dose was repeated on days four, five and six. The mice were killed and the ascites fluid collected on the seventh day after the original inoculation with ascites cells.

### *Empty Ribosomes*

The technique for the preparation of run-off *N*-ethylmaleimide-treated and recentrifuged ribosomes has been described in the previous paper [2] in this series. For convenience these will be referred to as empty ribosomes.

### *Preparation of Ribosomes with A-Site and P-Site Occupation*

*P-Site Ribosomes.* 100 $A_{260}$ units of washed run-off ribosomes were incubated 10 min at 30 °C with 100 μg EF-1 and 6 nmol [$^{14}$C]Phe-tRNA (400 dis. $\times$ min$^{-1}$ $\times$ pmol$^{-1}$) in a binding system as described previously [1]. 40 μg EF-2 was then added and the incubation continued for a further 30 min at 30 °C. This incubation mixture was then layered on top of 5.5 ml of 5% sucrose, 500 mM $NH_4Cl$, 30 mM Tris-HCl (pH 7.6 at 20 °C), 7 mM $Mg(CH_3COO)_2$, 1 mM dithioerythritol, which had been layered over 0.5 ml of 10% sucrose, 100 mM $NH_4Cl$, 20 mM Tris-HCl (pH 7.6 at 20 °C), 10 mM $Mg(CH_3COO)_2$ and 1 mM dithioerythritol. This was centrifuged for 14 h at 130000 $\times$ $g$ and the pellet dissolved in 200 mM sucrose, 100 mM $NH_4Cl$, 20 mM Tris-HCl (pH 7.6 at 20 °C), 10 mM $Mg(CH_3COO)_2$ and 1 mM dithioerythritol. It was demonstrated that 94% of the [$^{14}$C]phenylalanine bound to these ribosomes was puromycin-sensitive.

*A-Site Ribosomes.* The P-site ribosomes described above were the source for the preparation of A-site ribosomes. After incubation with EF-2, but before centrifugation, the ribosomes were exposed to 13 mM dithioerythritol (26 mM –SH) for 5 min at 30 °C, *N*-ethylmaleimide was then added to a final concentration of 41 mM for 10 min at 0 °C. Subsequently, excess *N*-ethylmaleimide was reacted with 46 mM 2-mercaptoethanol. 40 μg of EF-1 and 3000 pmol of [$^{14}$C]Phe-tRNA were added and incubated for 10 min at 30 °C. The resulting A-site ribosomes were then centrifuged as described above. They showed 72% A-site occupation.

### *Monosomes with Occupied P-Sites*

These particles were prepared in a similar manner to that described above except that ascites polysomes were used as the starting material. After the incubation, *N*-ethylmaleimide was added to inactivate EF-2 and

subsequently 2-mercaptoethanol was used to inactivate it as described above. Then 0.5 μg ribonuclease A was added for 10 min at 0 °C. These derived P-site monosomes were then centrifuged under conditions similar to those described above.

*Monosomes with Occupied A-Sites*

Baliga and Munro [4] have shown that, as normally isolated, approximately 80% of polysomes have nascent peptides in the A-site. Digestion of these polysomes with ribonuclease A and centrifugation as described above result in A-site monosomes.

*Ribosomal Subunits*

Ascites polysomes which had been centrifuged through 500 mM $NH_4Cl$, 30 mM Tris-HCl (pH 7.5 at 20 °C), 10 mM $Mg(CH_3COO)_2$ and 1 mM dithioerythritol were used for the preparation of subunits. These polysomes were dissociated into subunits according to the technique of Blobel and Sabatini [11].

*Elongation Factor 1*

The preparation of EF-1 has been described in an earlier paper from this laboratory [1].

*Elongation Factor 2*

This preparation was identical to that described for EF-1 up to the adsorption on calcium phosphate gel. The neutralised post-pH-5 supernatant from approximately 500 g of ascites cells was adsorbed on 180 g of calcium phosphate gel. After three washes with 0.3 M sucrose, 0.1 M KCl, 0.05 M Tris-HCl (pH 7.4), 1 mM dithioerythritol and 0.1 mM EDTA, the EF-2 was eluted by extracting three times with 250 ml 0.15 M phosphate buffer pH 6.8 and then concentrated by 80% $(NH_4)_2SO_4$ precipitation at pH 7.5 (4 °C). The pellet was dissolved in 14 ml of buffer A [50 mM Tris-HCl (pH 8.05 at 4 °C), 1 mM dithioerythritol and 0.1 mM EDTA] to give a final protein concentration of 23 mg/ml and extensively dialyzed against the same buffer. This was then layered on to a 5 × 8.5-cm column of DEAE-cellulose which had been carefully equilibrated (pH and conductivity checked) with the same buffer. The column was then washed with 350 ml of buffer A. The EF-2 was eluted with a linear gradient consisting of 350 ml of buffer A and 350 ml of 0.1 M KCl in buffer A. The majority of the biological activity emerged in the (0.08–0.1 KCl) concentration range of the gradient. Active fractions were pooled and precipitated with 80% $(NH_4)_2SO_4$. The pellet obtained was dissolved in 11.5 ml of buffer A at a protein concentration of 6 mg/ml and dialyzed for 16 h against two changes of this buffer. Sephadex G-150 chromatography of this material gave no increase in purity. Consequently the active Sephadex fractions (104 ml of 0.1 mg/ml) were applied to a 3 × 3.5-cm column of cellulose phosphate which had been extensively washed and equilibrated with buffer A. After the sample had been applied, the column was washed with approximately one liter of the same buffer. A large amount of protein (free of EF-2 activity) was removed by this wash. Elution with 100 ml of 50 mM KCl in buffer A was followed by elution with 250 ml of 100 mM KCl in buffer A. The majority of the EF-2 activity emerged with 100 mM KCl, but some activity was also recovered in the leading fractions of the 50 mM eluate. These two activity peaks were separately concentrated by vacuum dialysis against buffer A. They were further purified by sucrose gradient electrophoresis at 4 °C for 62 h using 1200 V at 2.5 mA in a similar manner to that described earlier for EF-1 [1].

*Assays of Elongation Factors*

EF-1 function was measured in the aminoacyl-tRNA binding assay described earlier [1].

EF-2 function was assayed in a protein synthesizing assay *in vitro* which differed from the conditions used to assay EF-1 in that a creatine phosphate and creatine kinase GTP regenerating system was always present. Hydrolysis of the products of synthesis was accomplished by incubating the samples in 10% trichloroacetic acid at 90 °C for 15 min or at 37 °C for 15 min in 0.4 N KOH. Proteins were precipitated by ice-cold 5% trichloroacetic acid and the precipitate collected on Whatman GF/B glass fibre filters for liquid scintillation counting.

*$^3$H-Labelled Factor Binding*

The binding of $^3$H-labelled EF-1 and EF-2 to ribosomes was studied by incubating ribosomal subunits or ribosomes of the appropriate type (usually 2 $A_{260}$ units) with one of the factors in the presence of 60 mM KCl, 10 mM $NH_4Cl$, 25 mM Tris-HCl (pH 7.9 at 20 °C), 5 mM dithioerythritol and 6 mM $Mg(CH_3COO)_2$ for 30 min at 30 °C. This mixture was then cooled and centrifuged directly as described below, or first fixed with glutaraldehyde. When this fixation was used, 30 μl of a 15 mg/ml albumin solution was added prior to making the incubation mixture 1% in ice-cold neutralised glutaraldehyde. This is a modification of the technique of Subramanian [12]. The samples were layered on ice-cold 5–16% sucrose gradient containing 80 mM KCl, 30 mM Tris-HCl

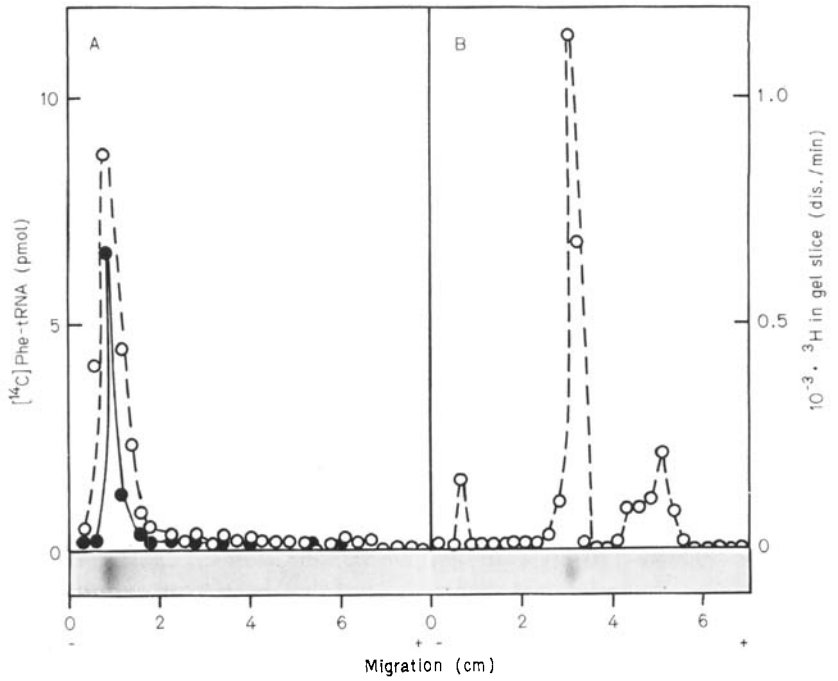


Fig. 1. *Polyacrylamide gel electrophoresis of purified [$^3$H]EF-1.* (A) 3% acrylamide gels with 9 µg [$^3$H]EF-1 on each gel were run for 15 h at 0 °C using 5 mA per gel. Three identical gels were run simultaneously. The first was stained with Coomassie brilliant blue, to visually locate the protein bands. The second was transversely sliced into 2-mm slices, these were incubated with 200 µl 30% $H_2O_2$ for 20 h in sealed scintillation vials at 55 °C; after cooling, 10 ml of Packard Instagel was added to each vial and they were assayed for $^3$H activity (○). The third gel was also sliced in the same way, but each slice was eluted overnight with 50 µl of buffer containing 1 mg human albumin per ml. The eluates were then assayed for [$^{14}$C]Phe-tRNA binding capacity in the standard binding system (●). (B) 7% acrylamide gels were made 0.1% in sodium dodecylsulphate and run at 10 mA per gel for 3 h at 15 °C. One gel was stained and the other was sliced and $^3$H activity counted as described above

(pH 7.6 at 20 °C) and 6 mM $Mg(CH_3COO)_2$ in a 17-ml tube of the Spinco SW 27 rotor. Centrifugation was carried out at 4 °C for 3 h at 27000 rev./min.

After centrifugation, the gradients were pumped through an LKB uvicord ultraviolet scanner and the absorbance was recorded. Fractions (0.9 ml) were collected directly in scintillation vials and 10 ml of Packard Insta-Gel scintillation fluid was added. The samples were counted in a Packard 3380 scintillation counter adjusted for simultaneous determination of $^{14}$C and $^3$H. For the quantitative assessment of radioactive elongation factors bound to ribosomes the following procedure was followed: the radioactivity associated with each fraction was converted to picomoles and plotted. The total amount of factor bound to ribosomes was then calculated by summing all values from the first fraction of the leading edge of the radioactivity peak to the last value which still fell within the trailing edge of the 80-S ribosomal peak.

### *Acrylamide Gel Electrophoresis*

The gels were made according to the same procedure as described earlier [1].

### *Extraction of Biological Activity from Acrylamide Gels*

After electrophoresis, the acrylamide gels were sliced into 2-mm slices (in the case of 3% gels it was necessary to freeze the gels on a sheet of glass laid on a solid $CO_2$ block, before slicing). Gel slices were then eluted in a manner similar to that described by Collins *et al.* [13] by putting each slice in a test tube containing 50 µl of 300 mM sucrose, 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 1 mg/ml human serum albumin, 0.1 mM EDTA and 1 mM dithioerythritol (for EF-2, the concentration of dithioerythritol was raised to 5 mM). The slices were left for 16 h at 4 °C in an atmosphere of nitrogen and 5 µl of each sample was assayed for either EF-1 or EF-2 activity.

All other methods were as described previously [1,2].

## RESULTS

### *Purity of Labelled Elongation Factors*

The data obtained from an analysis of [$^3$H]EF-1 on three acrylamide gels run in parallel are presented

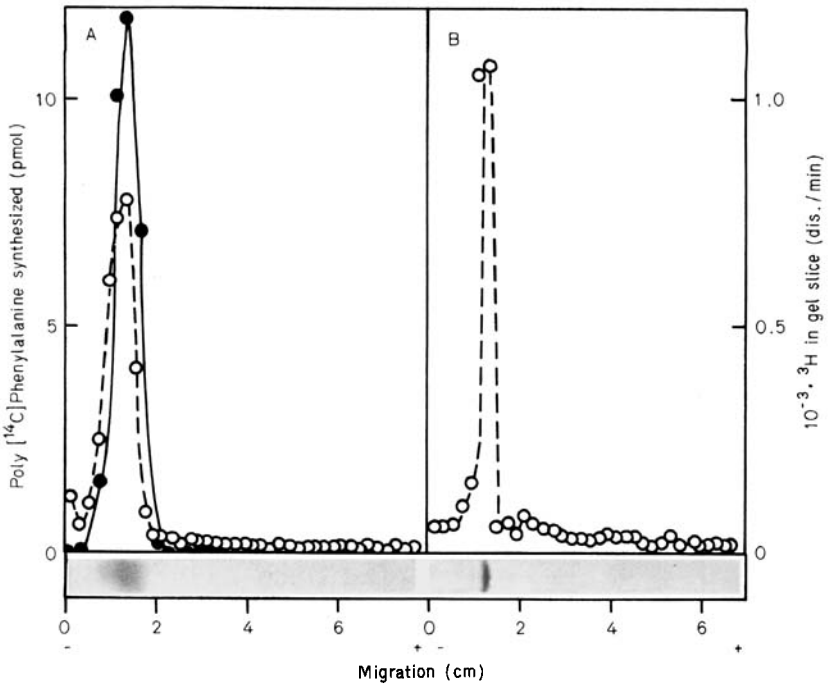


Fig. 2. *Polyacrylamide gel electrophoresis of purified [$^3$H]EF-2.* (A) 6% acrylamide gels were run at 10 mA for 15 h at 0 °C. As in Fig. 1(A) three gels were run in parallel. In order to stabilize EF-2, cysteine (5 mM) was added to the buffers and 5 mM dithioerythritol was added to the eluting buffer for activity assays. (○) $^3$H radioactivity; (●) poly(phenylalanine) synthesising activity in the standard synthesis assay. (B) Dodecyl sulphate gels of [$^3$H]EF-2 run under the same conditions as described for Fig. 1(B)

in Fig. 1A. It is obvious that all the protein migrated as a single band and that all the radioactivity as well as biological activity were associated with this one component. A radioactivity scan of a dodecylsulphate-acrylamide gel (Fig. 1B) revealed the presence of some radioactivity apart from the main band, corresponding to a molecular weight of 47000, which is characteristic for EF-1 [1]. From these data it was concluded that the [$^3$H]EF-1 preparation used in this study contained at least 70% of the 47000-$M_r$ subunit.

[$^3$H]EF-2 was analysed in a similar fashion and the results are depicted in Fig. 2. In order to protect the −SH groups of EF-2 against oxidation, the blank gels were electrophoresed for 2 h with electrode buffers made 5 mM in cysteine. This pretreatment removed residual traces of ammonium persulphate and made the gels approximately 5 mM in cysteine. (Cysteine added directly to the acrylamide, prior to polymerisation, would interfere with the polymerisation process and would be partly or totally oxidised by the sodium persulphate catalyst used to promote polymerisation.) [$^3$H]EF-2 samples were then applied to the gels and electrophoresed, again using cysteine-containing electrode buffers. As can be seen from Fig. 2 (A), the biological activity under these conditions was recovered in high yield and coincided precisely with the only radioactive peak of the gel. Similarly, there was coincidence with the only protein on the gel as visualised by staining. Dodecylsulphate-acrylamide gel electrophoresis of this material also showed a single stained band of protein and an identically located single band of radioactivity (Fig. 2B). It was concluded from these data that the [$^3$H]EF-2 used in these studies was more than 95% pure.

### *Conditions Required for the Binding of EF-1 and EF-2 to Ribosomes and Subunits*

Due to the fact that labelled EF-1 has not been available in pure form, the nature of the events occurring during ribosome/factor interaction have not been amenable to study. It had previously been demonstrated that in order for EF-1 to bind aminoacyl-tRNA to ribosomes, GTP (or Guo-5′-$P_2$-$CH_2$-$P$), poly(uridylic acid) (or messenger RNA) and aminoacyl-tRNA are essential [14]. Until now, however, it has not been clarified whether EF-1, after it has functioned, remains bound to the ribosome or is immediately released. When a reaction mixture containing [$^3$H]EF-1, [$^{14}$C]Phe-tRNA, poly(uridylic acid), GTP and ribosomes was centrifuged on a sucrose gradient, significant [$^{14}$C]Phe-tRNA attachment to

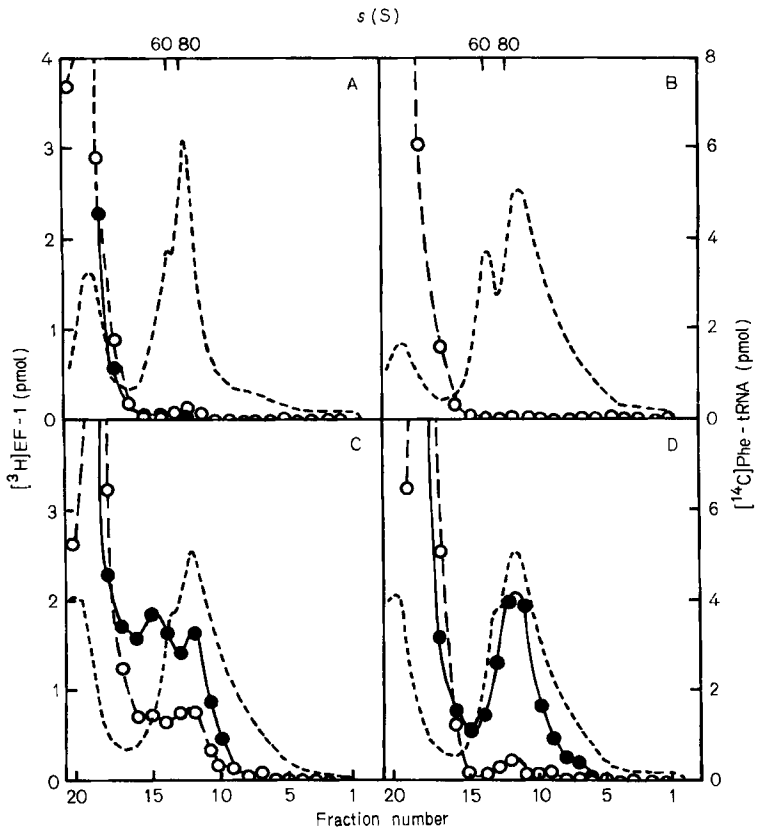


Fig. 3. *[$^3$H]EF-1 binding to empty ribosomes.* 0.1-ml reaction mixtures were incubated for 30 min at 30 °C and contained 70 mM sucrose, 60 mM KCl, 35 mM $NH_4Cl$, 30 mM Tris-HCl (pH 7.9 at 20 °C), 4.4 mM dithioerythritol, 5.5 mM $Mg(CH_3COO)_2$, 7.7 $A_{260}$ units of empty ribosomes and 6 µg [$^3$H]EF-1 (184 dis. × min$^{-1}$ × pmol$^{-1}$), in addition to the components indicated below for the individual panels. (A) Guo-5′-$P_2$-$CH_2$-$P$ (0.2 mM); [$^{14}$C]Phe-tRNA (*E. coli*, 173 dis. × min$^{-1}$ × pmol$^{-1}$), 55 pmol. (B) Guo-5′-$P_2$-$CH_2$-$P$ (0.2 mM); poly(uridylic acid), 40 µg. (C) Guo-5′-$P_2$-$CH_2$-$P$ (0.2 mM); poly(uridylic acid), 40 µg; [$^{14}$C]Phe-tRNA (55 pmol). (D) GTP (0.2 mM); poly(uridylic acid), 40 µg; [$^{14}$C]Phe-tRNA (55 pmol). The samples were layered on 17-ml 5–16% linear sucrose gradients in 80 mM KCl, 30 mM Tris-HCl (pH 7.9 at 20 °C) and 6 mM $Mg(CH_3COO)_2$. Centrifugation was for 3 h at 27000 rev./min in the SW 27 rotor at 4 °C. Absorbance at 260 nm was recorded continuously and 0.8-ml fractions were collected for simultaneous $^3$H and $^{14}$C radioactivity determination. (O----O) [$^3$H]EF-1; (●——●) [$^{14}$C]Phe-tRNA; (------) absorbance at 260 nm

the ribosomes was observed. However, no $^3$H activity was visible in the region of the ribosomal peak (Fig. 3D). Thus, although the factor had functioned, it must have been released after the attachment of Phe-tRNA$^{Phe}$. A similar failure to detect ribosome-associated [$^3$H]EF-1 was encountered when ribosomes, [$^3$H]EF-1, poly(uridylic acid), aminoacyl-tRNA and GTP were mixed in combination or with any one or more components omitted (results not shown).

Significant [$^3$H]EF-1 binding to ribosomes could only be detected in a complete aminoacyl-tRNA binding system in which GTP had been replaced by the non-hydrolysable analogue, Guo-5′-$P_2$-$CH_2$-$P$ (Fig. 3C). When poly(uridylic acid) or aminoacyl-tRNA were omitted from this system again no binding of EF-1 to ribosomes could be observed. These results are depicted in Fig. 3 (A) and (B).

Fig. 4 shows that the trailing of the [$^3$H]EF-1 binding profile can be eliminated by fixing the reaction mixture with 1% (v/v) glutaraldehyde prior to centrifugation. This experiment and other experiments in which glutaraldehyde fixation was used confirmed the specificity of the EF-1/ribosome interaction with respect to the requirements for Phe-tRNA$^{Phe}$, poly(uridylic acid) and Guo-5′-$P_2$-$CH_2$-$P$. Since the two techniques provided qualitatively identical results and the use of glutaraldehyde with labelled EF-2 was found unnecessary, only the results obtained without glutaraldehyde fixation are presented in the following pages.

Culp *et al.* [15] reported that aminoacyl-tRNA binding to ribosomes at 0 °C in the presence of GTP was similar to that observed at 37 °C in the presence of Guo-5′-$P_2$-$CH_2$-$P$. In contrast, we were unable to

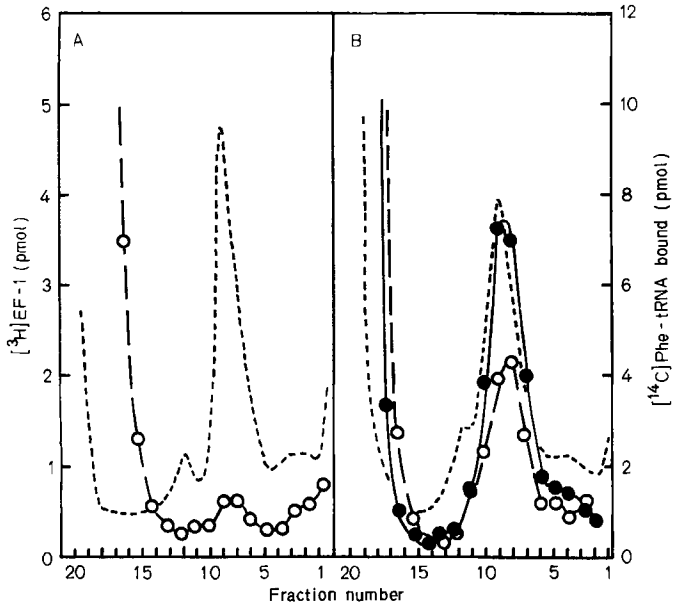


Fig. 4. *[$^3$H]EF-1 binding to empty ribosomes with glutaraldehyde fixation.* Incubations were as described in Fig. 3. (A) Guo-5′-$P_2$-$CH_2$-$P$ (0.2 mM). (B) Guo-5′-$P_2$-$CH_2$-$P$ (0.2 mM); poly(U) (40 µg); [$^{14}$C]Phe-tRNA (*E. coli*, 173 dis. × min$^{-1}$ × pmol$^{-1}$), 55 pmol. After incubation the tubes were cooled on ice, 30 µl of 15 mg/ml albumin solution was added, then 30 µl of 5% neutralized glutaraldehyde in 7 mM KCl, 200 mM Tris-HCl (pH 7.6 at 20 °C) and 3.5 mM $Mg(CH_3COO)_2$ was added. Gradients were centrifuged and collected as described for Fig. 3. (O——O) [$^3$H]EF-1; (●——●) [$^{14}$C]Phe-tRNA; (------) absorbance at 260 nm

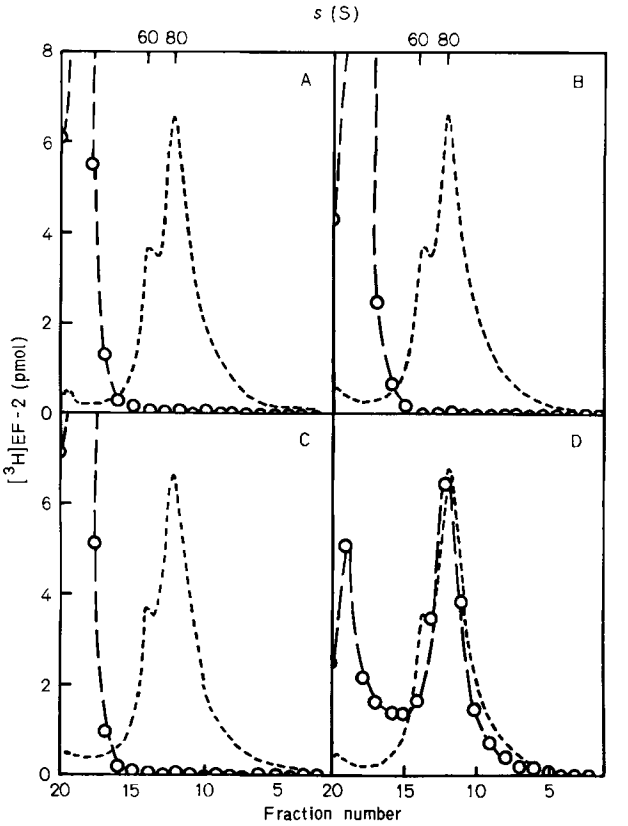


Fig. 5. *[$^3$H]EF-2 binding to empty ribosomes.* Assays similar to those described for Fig. 3 contained 7 µg of [$^3$H]EF-2 (178 dis. × min$^{-1}$ × pmol$^{-1}$) instead of [$^3$H]EF-1. (A) No additions. (B) GDP (0.2 mM). (C) GTP (0.2 mM). (D) Guo-5′-$P_2$-$CH_2$-$P$ (0.2 mM). (O——O) [$^3$H]EF-2; (------) absorbance at 260 nm

observe [$^3$H]EF-1 binding at 0 °C in the presence of GTP (data not shown). The properties of the EF-1/ribosome interaction were further characterised by demonstrating that Met-tRNA$^{Met}$ (ascites) and deacylated Phe-tRNA$^{Phe}$ only permitted low [$^3$H]EF-1 binding to ribosomes programmed by poly(uridylic acid), under the same conditions under which Phe-tRNA promoted good attachment of EF-1 (data not shown). These data show that both codon-anticodon recognition and acylation of Phe-tRNA are required for the binding of EF-1 to ribosomes.

It was found that EF-2 also binds to ribosomes only in the presence of Guo-5′-$P_2$-$CH_2$-$P$ (Fig. 5D). In the presence of this nucleotide analogue, firm attachment of the factor to empty ribosomes was observed. No stable association between EF-2 and ribosomes appeared to occur in the absence of a nucleotide or with GDP or GTP (Fig. 5A, B and C). [$^3$H]EF-2 binding experiments were also performed at 0 °C, under which conditions, identical results to those observed at 30 °C were obtained.

Neither EF-1 nor EF-2 became attached to 40-S or 60-S subunits when incubated with these particles under conditions which permit binding of the elongation factors to 80-S ribosomes (Table 1). However, a mixture of both subunits exhibits binding characteristics identical with those observed in 80-S ribosomes. This finding implies that the binding sites of both factors are only incompletely represented on either subunit and that only the intact 80-S ribosome can serve as a substrate for the attachment of EF-1 and EF-2.

### *Mutual Effects of Factors upon One Another*

Experiments carried out in this and in other laboratories led to the inference that EF-1 and EF-2 bind to the same region of the ribosome and therefore interfere with one another [3, 5, 16]. This suggestion was confirmed in the present study. When [$^3$H]EF-1 and [$^{14}$C]Phe-tRNA$^{Phe}$ were bound to the ribosome in the presence of poly(uridylic acid) and Guo-5′-$P_2$-

Table 1. *Interaction of $^3H$-labelled elongation factors with ribosomal subunits and ribosomes of different types*
0.1-ml samples contained 0.2 mM Guo-5′-$P_2$-$CH_2$-$P$, 80 mM sucrose, 60 mM KCl, 25 mM $NH_4Cl$, 25 mM Tris-HCl (pH 7.9), 4 mM dithioerythritol and 6 mM $Mg(CH_3COO)_2$. 40-S subunits, 60-S subunits and empty ribosomes were used at 0.53 $A_{260}$ unit, 1.08 $A_{260}$ unit or 2.2 $A_{260}$ units respectively. 2.61 $A_{260}$ units of A-site ribosomes, 2.4 $A_{260}$ units of P-site ribosomes and 8 $A_{260}$ units of both A-site and P-site monosomes were used. 23 pmol of [$^3$H]EF-1 and 35 pmol of [$^3$H]EF-2 were present when indicated. 55 pmol of [$^{14}$C]Phe-tRNA$^{Phe}$ was added with [$^3$H]EF-1. 40 µg of poly(uridylic acid) was added when subunits or empty ribosomes were examined for EF-1 binding capacity. Incubations were carried out at 30 °C for 30 min. Sucrose gradient analysis and assays for radioactivity were as described under Methods. Results are presented as pmol of substrate bound per nmol of ribosomes (or subunits)

| Particle | [$^3$H]EF-1 | [$^{14}$C]Phe-tRNA | [$^3$H]EF-2 |
|---|---|---|---|
| 40-S subunits | 1.25 | 21.0 | – |
| | – | – | 10.0 |
| 60-S subunits | 14.0 | 31.0 | – |
| | – | – | 12.0 |
| 40-S + 60-S subunits | 69.0 | 270.0 | – |
| | – | – | 250.0 |
| Empty ribosomes | 68.0 | 240.0 | – |
| | – | – | 250.0 |
| A-site ribosomes | 48.0 | ≈190 | – |
| A-site monosomes | – | – | 161.0 |
| P-site ribosomes | 150.0 | ≈420 | – |
| P-site monosomes | – | – | 82.0 |

$CH_2$-$P$ and unlabelled EF-2 was then added for a second incubation period, part of both the prebound EF-1 and the Phe-tRNA$^{Phe}$ were removed from the ribosomal A-site (Fig. 6, Table 2). A preincubation of ribosomes with unlabelled EF-2 and Guo-5′-$P_2$-$CH_2$-$P$ decreases the subsequent attachment of [$^3$H]-EF-1 and [$^{14}$C]Phe-tRNA even more drastically (Table 2). These observations provide direct evidence for mutual, or at least overlapping, binding sites of the two factors on 80-S ribosomes. The fact that prebound [$^3$H]EF-1 is partially removed from the ribosome by a subsequent incubation with EF-2 implies that the affinity of the common factor-binding region for EF-2 is higher than for EF-1.

The close spatial relationship of the EF-1 and EF-2 binding sites is also reflected by the results of experiments in which the influence of the attachment of [$^3$H]EF-2 to ribosomes was studied. Preincubation of ribosomes with EF-1, Phe-tRNA and poly(uridylic acid) caused only little inhibition of [$^3$H]EF-2 binding as compared to parallel assays in which ribosomes had only been preincubated with poly(uridylic acid) and Phe-tRNA (Table 3). Surprisingly, the attachment of [$^3$H]EF-2 was even stimulated when this

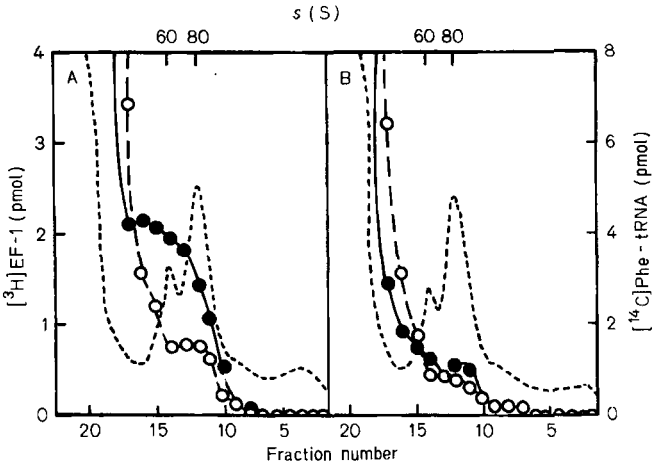


Fig. 6. *Displacement of prebound [$^3$H]EF-1 and [$^{14}$C]Phe-tRNA from ribosomes by unlabelled EF-2.* (A) First incubation conditions as in Fig. 3C. The second incubation lasted for 30 min at 30 °C. (B) First incubation as above. Second incubation: 17 µg of purified EF-2 was added and incubated for a further 30 min at 30 °C. Centrifugation and analysis were as described for Fig. 3. (O——O) [$^3$H]EF-1; (●——●) [$^{14}$C]Phe-tRNA; (------) absorbance at 260 nm

Table 2. *The influence of EF-2 on the binding of [$^3$H]EF-1 under various conditions*
The preincubation was for 30 min at 30 °C. 0.1-ml volumes contained empty ribosomes (2 $A_{260}$ units); poly(uridylic acid), 40 µg; [$^{14}$C]Phe-tRNA (*E. coli*, 173 dis. × min$^{-1}$ × pmol$^{-1}$), 50 pmol; Guo-5′-$P_2$-$CH_2$-$P$ (0.2 mM) and the additional components indicated in the preincubation column. 9.0 µg of [$^3$H]EF-1 and 8.5 µg EF-2 were introduced where indicated. The second incubation was also for 30 min at the same temperature. Results are presented as pmol substrate bound per nmol ribosomes

| Preincubation | 2nd incubation | [$^3$H]EF-1 bound | [$^{14}$C]Phe-tRNA bound |
|---|---|---|---|
| *Expt 1* | | | |
| – | [$^3$H]EF-1 | 75.0 | 260 |
| + EF-2 | [$^3$H]EF-1 | 17.5 | 42.5 |
| *Expt 2* | | | |
| [$^3$H]EF-1 | – | 62.5 | 240 |
| [$^3$H]EF-1 | EF-2 | 42.5 | 90 |

factor was introduced during a preincubation period and EF-1 as well as the components necessary for its interaction with the ribosome were added subsequently (Table 3, Expt 2; Fig. 7).

### *Effect of Showdomycin on the Binding of Elongation Factors to Ribosomes*

Showdomycin has been reported to inhibit the formation of the EF-2 · ribosome complex and also to interfere with nonenzymatic or EF-1-directed

Table 3. *The influence of EF-1 on the binding of [$^3$H]EF-2 under various conditions*
Ribosomes (2.2 $A_{260}$ units) and Guo-5′-$P_2$-$CH_2$-$P$ (0.2 mM) were present with the components indicated during 30 min preincubation at 30 °C. 40 μg of poly(uridylic acid), 50 pmol of [$^{14}$C]Phe-tRNA (*E. coli*, 173 dis. × min$^{-1}$ × pmol$^{-1}$), 20 μg of purified EF-1 and 7.3 μg of [$^3$H]EF-2 were used where indicated. The second incubation was for 30 min also at 30 °C. The addition of the components for the second incubation lowered the $Mg^{2+}$ concentration to 4.5 mM during this period. Results are presented as pmol substrate bound per nmol ribosomes

| Preincubation | 2nd incubation | [$^3$H]EF-2 bound | [$^{14}$C]Phe-tRNA bound |
|---|---|---|---|
| *Expt 1* | | | |
| Poly(U) + [$^{14}$C]Phe-tRNA | [$^3$H]EF-2 | 224 | 15.9 |
| Poly(U) + EF-1 + [$^{14}$C]Phe-tRNA | [$^3$H]EF-2 | 175 | 100 |
| *Expt 2* | | | |
| [$^3$H]EF-2 | [$^{14}$C]Phe-tRNA | 150 | 0.91 |
| [$^3$H]EF-2 | poly(U) + EF-1 + [$^{14}$C]Phe-tRNA | 296 | 96.5 |

binding of aminoacyl-tRNA to ribosomes [17,18]. These findings have been taken as indirect evidence for overlapping binding sites of EF-1 and EF-2 on the ribosome. The availability of labelled elongation factors made it possible to assess the effect of showdomycin on the binding of these two factors more directly. As depicted in Fig. 8 (A and B), showdomycin clearly inhibits the attachment of EF-1 and aminoacyl-tRNA to ribosomes. At the same time, it can be seen from Fig. 8 (C and D) that the binding of labelled EF-2 is also inhibited by this antibiotic although not as drastically as the attachment of EF-1. Similar inhibition of the binding of both factors was obtained with N-ethylmaleimide (results not shown).

### *The Binding of Labelled Elongation Factors to A- and P-Site Ribosomes*

In a previous communication from this laboratory [3] as well as in reports from other laboratories [6,19] evidence was presented which appeared to indicate that A-site ribosomes are the proper substrates for the binding of EF-2, while the binding of EF-1 occurred preferentially or exclusively on P-site ribosomes. The data summarised in the lower part of

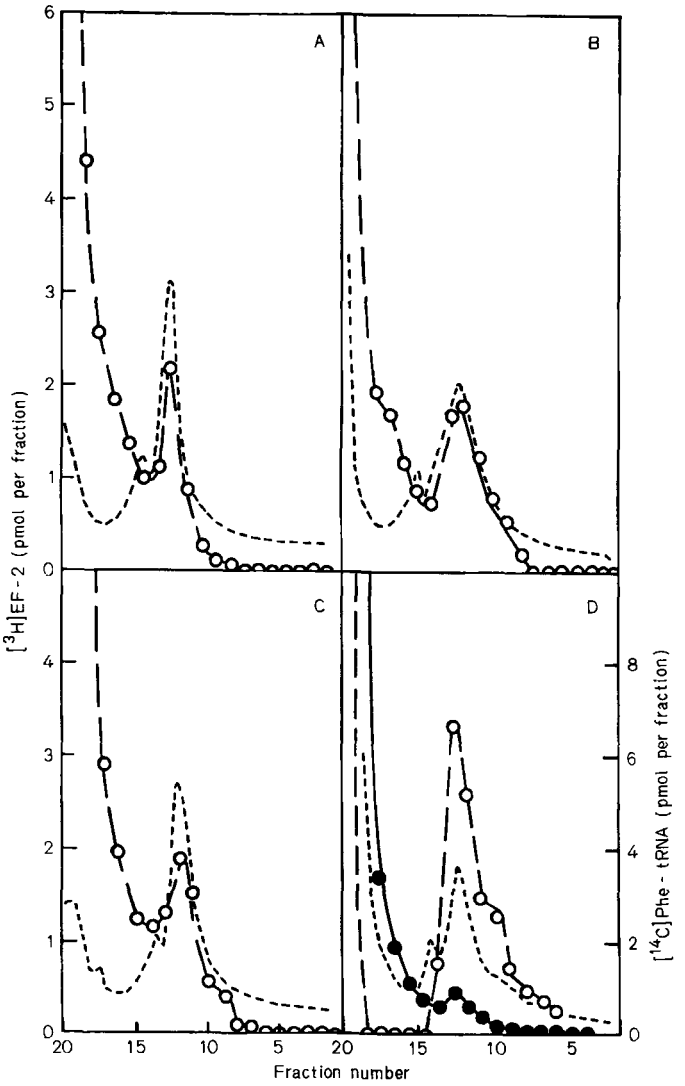


Fig. 7. *Enhancement of [$^3$H]EF-2 binding by addition of EF-1 and [$^{14}$C]Phe-tRNA in a post-incubation.* First incubations (30 min at 30 °C) contained 2.2 $A_{260}$ units of empty ribosomes, 7.3 μg of [$^3$H]EF-2, 0.2 mM Guo-5′-$P_2$-$CH_2$-$P$ and sucrose and salts as indicated in the legend to Fig. 3. In the second incubations the additions indicated below were made, as well as a compensating salt solution adjusted to maintain the ionic conditions prevailing during the first incubation. (A) No addition. (B) 40 μg poly(uridylic acid). (C) 20 μg EF-1. (D) 40 μg poly(uridylic acid), 20 μg EF-1 and 55 pmol [$^{14}$C]Phe-tRNA (*E. coli*, 173 dis. × min$^{-1}$ × pmol$^{-1}$). (O- - -O) [$^3$H]EF-2; (●——●) [$^{14}$C]Phe-tRNA; (------) absorbance at 260 nm

Table 1 provide direct confirmation of this view. P-site ribosomes are good substrates for the attachment of EF-1. P-site monosomes are relatively poor substrates for the binding of EF-2 while the opposite is true for A-site occupied particles. The [$^3$H]EF-1 binding exhibited by the A-site ribosomes may be attributable to contamination by P-site occupied ribosomes. Similarly, contamination of the P-site

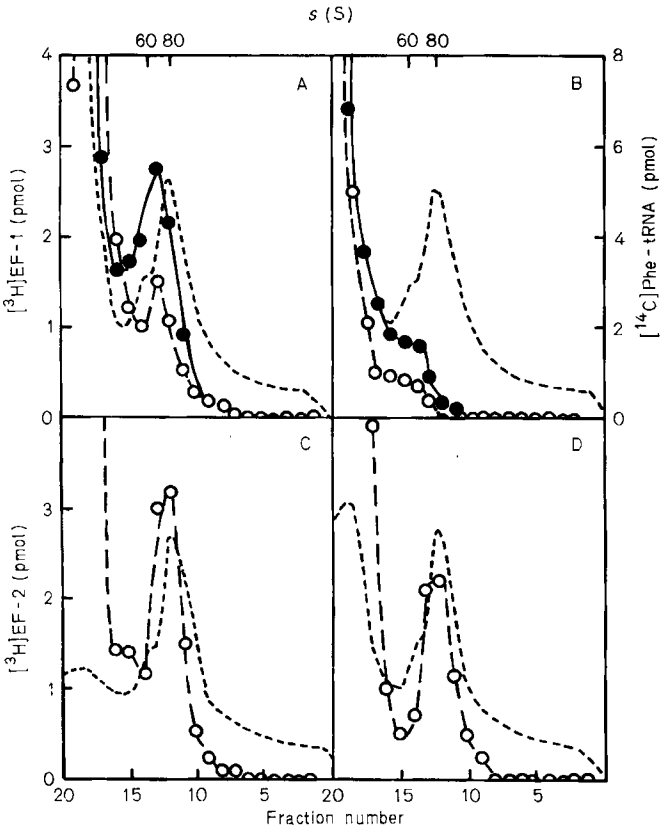


Fig. 8. *Effect of showdomycin on [$^3$H]EF-1 and [$^3$H]EF-2 binding to empty ribosomes.* Ribosomes (2.2 $A_{260}$ units) and 0.2 mM Guo-5′-$P_2$-$CH_2$-$P$ were preincubated for 10 min at 30 °C under the ionic conditions described in Fig. 3. In addition the preincubation mixtures contained: (A) poly(uridylic acid), 40 µg. (B) poly(uridylic acid), 40 µg, plus 10 mM showdomycin. (C) No addition. (D) 10 mM showdomycin. Subsequently the following additions were made: (A) and (B): 8.9 µg [$^3$H]EF-1 and 60 pmol [$^{14}$C]Phe-tRNA (*E. coli*, 173 dis. × min$^{-1}$ × pmol$^{-1}$). (C) and (D): 28 pmol [$^3$H]EF-2. The second incubations lasted 30 min at 30 °C. Centrifugation and analysis as described for Fig. 3. (O----O) [$^3$H]EF-1 or [$^3$H]EF-2; (●——●) [$^{14}$C]Phe-tRNA; (------) absorbance at 260 nm

monosomes by A-site monosomes may explain the former's capacity to bind some EF-2. We therefore conclude, that, although empty ribosomes can bind either of the two elongation factors, there is at least a strong tendency for A-site particles to associate with EF-2 and for the P-site occupied species to bind EF-1.

## DISCUSSION

The availability of highly purified radioactive elongation factors from mouse ascites cells has made it possible for the first time to directly study the interaction of EF-1 and EF-2 with ribosomes and to define the conditions for their attachment to, and their release from, ribosomes. EF-1 can only bind to the ribosomes if: (a) the ribosome has been programmed with a polynucleotide, (b) the aminoacyl-tRNA, coded for by the polynucleotide, is available and (c) a guanosine moiety carrying three phosphate groups is present.

Under the conditions we have used, a direct assessment of EF-1 binding to ribosomes is only possible in the presence of the GTP analogue in which the γ-phosphate is nonhydrolyzable. It is obvious that EF-1 interacts with ribosomes in the presence of GTP and poly(uridylic acid), since Phe-tRNA$^{Phe}$ becomes attached to the ribosome under these conditions; however, this interaction appears to be an extremely transient one. The replacement of GTP by Guo-5′-$P_2$-$CH_2$-$P$ inhibits the release of EF-1. It is suggested, therefore, that GTP hydrolysis may provide the impetus which enables EF-1 to leave the ribosome. This mechanism would be in strict analogy to the corresponding steps in bacterial systems [20,21].

In most of our experiments the radioactivity profiles representing ribosome-associated [$^3$H]EF-1 and [$^{14}$C]Phe-tRNA displayed smearing. This phenomenon which can be completely avoided by glutaraldehyde fixation, indicates that a certain proportion of EF-1 molecules and Phe-tRNA dissociated from the ribosomes in the course of centrifugation. We conclude from this finding that the complex comprising the ribosome, Phe-tRNA$^{Phe}$, EF-1 and Guo-5′-$P_2$-$CH_2$-$P$ is not stable. The fact that the [$^{14}$C]Phe-tRNA when bound to ribosomes in the presence of GTP instead of Guo-5′-$P_2$-$CH_2$-$P$ gives a sharp radioactivity peak suggests that the Phe-tRNA becomes more firmly attached to the ribosomal A-site after GTP cleavage and ejection of EF-1. This would infer that after EF-1 release, the region of aminoacyl-tRNA which had interacted with EF-1 is now available to further stabilize its attachment to the ribosome. Earlier experiments from this laboratory showed that EF-1 interacts with the aminoacyl ester region of the aminoacyl-tRNA [2]. Hence, an EF-1 · aminoacyl-tRNA · ribosome complex should be unable to participate in peptide bond formation; this complex would be analogous to the entry site complex proposed by Hardesty *et al.* [22]. These various observations lead to the conclusion that the amino acid region of the aminoacyl-tRNA must be ribosome-bound in order to provide stable attachment of aminoacyl-tRNA to the ribosome.

EF-2 can be bound to ribosomes only in the presence of Guo-5′-$P_2$-$CH_2$-$P$; the failure of GDP and GTP to function in this reaction again points to the conclusion that the energy released by GTP hydrolysis is coupled to eject the elongation factor from the ribo-

some. Like EF-1, EF-2 only binds in the presence of both ribosomal subunits. This suggests that both factor binding sites are partly determined by the 60-S and partly by the 40-S subunit.

In contrast to our observations, Traugh and Collier [23] had earlier reported an interaction between EF-2 and the 60-S ribosomal subunit derived from reticulocytes. These authors had used either EDTA or 2.2 M urea as the dissociating agents to produce their subunits. Both of these processes lead to extensive unfolding of the ribonucleoprotein and complete loss of activity of the subunits. In our study it was found that the combined subunits were capable of binding both EF-2 and EF-1 in a manner similar to empty ribosomes, thus attesting to their biological integrity. Smulson and Rideau [24] also could not detect a binding of EF-2 to ribosomal subunits. Rao and Moldave [25] reported that rat liver 40-S subunits are capable of interacting with EF-1 in the presence of aminoacyl-tRNA and poly(uridylic acid); a guanosine nucleotide was not required for this interaction. Their EF-1, however, was impure. Furthermore, the conditions used by these authors for the assessment of the binding of EF-1 to 40-S subunits (pH 8 at 4 °C, 10 mM $MgCl_2$, no monovalent cations) may have been sufficiently different from ours to permit interactions which we could not detect.

Information concerning the topographical relation between the binding sites of EF-1 and EF-2 has also emerged from studies which examined the mutual interference of the factors on each others binding to ribosomes. The addition of EF-2 after prebinding of [$^3$H]EF-1 and [$^{14}$C]Phe-tRNA$^{Phe}$ led to a significant loss not only of [$^3$H]EF-1 but also of [$^{14}$C]Phe-tRNA. This finding suggested that the two factors have a mutual binding site on the ribosomes or at least that their binding sites overlap. It also indicates that the affinity of the ribosome for EF-2 is significantly higher than for EF-1 as was also indicated by the binding profiles of the two factors (Fig. 3, 4 and 5). The observation that prebound EF-1 causes only a modest reduction on the subsequent attachment of EF-2 appears to be in line with this conclusion. The mutual interference of elongation factor binding in mammalian ribosomes has been suggested previously by a number of authors [5, 10] and also seems to occur in bacteria. Although the attachment of EF-2 precludes any stable interaction between EF-1 and the ribosomal surface, it does not completely prevent EF-1 from depositing aminoacyl-tRNA in the A-site. This finding may indicate, that EF-1 interacts with that part of the ribosome population which has not already bound EF-2. The results, however, do demonstrate that even relatively high concentrations of EF-1 are incapable of removing pre-bound EF-2 from ribosomes; in marked contrast to the effects observed when the sequence of addition of the factors is reversed. The strong preference exhibited by A-site and P-site ribosomes for the binding of EF-2 and EF-1 respectively is in line with previous observations made in this laboratory [3] and with results presented by Nombela and Ochoa for ribosomes from artemia salina [6] and by Modolell *et al.* for bacterial ribosomes [19]. Taken together with the fact that in the presence of GTP, the interaction of both factors with the ribosome is a very transient one, the results imply that a mutual interference of the two elongation factors with respect to their binding and function does not occur under conditions *in vivo*, as was also concluded by Baliga *et al.* [7].

The antibiotic showdomycin, which is a maleimide derivative, interferes with the binding of EF-1 and to a lesser extent with that of EF-2. One interpretation of this observation is that its site of action is in the region of the ribosome surface common to both factor binding sites. However, such specificity need not necessarily be invoked since it is known that this antibiotic is an −SH reagent [26] which may simply interfere with critical −SH groups in both sites. The fact that the effects of showdomycin can be mimicked by *N*-ethylmaleimide would favour the latter interpretation. Bermek *et al.* [17] and Bermek and Matthaei [18] have also reported similar multiple site inhibition by this antibiotic.

The use of pure labelled transfer factors enables some estimates of the stoichiometric relation between the number of bound factor and aminoacyl-tRNA molecules to be made. On the basis of evidence presented earlier [1] the [$^3$H]EF-1 used in these studies which had a molecular weight of 190000 may be considered to be an aggregate of four subunits, each of molecular weight 47000. In all our experiments the ratio of the number of phenylalanyl-tRNA to EF-1 molecules, bound to ribosomes, has consistently been approximately 4. This is surprising since a recycling of EF-1 should not have occurred in the presence of Guo-5′-$P_2$-$CH_2$-$P$. Hence the possibility that in the presence of ribosomes the factor dissociates into four active monomers each of which catalyzes the binding of 1 molecule of Phe-tRNA$^{Phe}$ to the ribosome, must now be seriously considered. Studies to test this possibility are in progress.

The results presented in this paper have described the biochemical events leading to the attachment of the two elongation factors to eukaryotic ribosomes. The data, however, provide no information regarding the subsequent fate of EF-1 upon its release from the ribosome. Experiments are now in progress to explore this problem.

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## REFERENCES

1. Drews, J., Bednarik, K. & Grasmuk, H. (1974) *Eur. J. Biochem. 41*, 217–227.
2. Nolan, R. D., Grasmuk, H., Högenauer, G. & Drews, J. (1974) *Eur. J. Biochem. 45*, 601–609.
3. Grasmuk, H., Nolan, R. D. & Drews, J. (1974) *Eur. J. Biochem. 48*, 485–493.
4. Baliga, P. S. & Munro, H. N. (1971) *Nat. New Biol. 233*, 257–258.
5. Okata, T. & Kaji, A. (1973) *Eur. J. Biochem. 38*, 46–53.
6. Nombela, C. & Ochoa, S. (1973) *Proc. Natl Acad. Sci. U.S.A. 70*, 3556–3560.
7. Baliga, B. S., Schechtman, M. G. & Munro, H. N. (1973) *Biochem. Biophys. Res. Commun. 51*, 406–413.
8. Richter, D. (1972) *Biochem. Biophys. Res. Commun. 46*, 1850–1856.
9. Bodley, J. W. & Lin, L. (1970) *Nature (Lond.) 227*, 60–62.
10. Okura, A., Kinoshita, T. & Tanaka, N. (1970) *Biochem. Biophys. Res. Commun. 41*, 1545–1550.
11. Blobel, G. & Sabatini, D. (1971) *Proc. Natl Acad. Sci. U.S.A. 68*, 390–394.
12. Subramanian, A. R. (1972) *Biochemistry, 11*, 2710–2714.
13. Collins, J. F., Moon, H. M. & Maxwell, E. S. (1972) *Biochemistry, 11*, 4187–4194.
14. Arlinghaus, R., Schaeffer, J. & Schweet, R. (1964) *Proc. Natl Acad. Sci. U.S.A. 51*, 1291–1299.
15. Culp, W., Odom, O. W. & Hardesty, B. (1973) *Arch. Biochem. Biophys. 155*, 225–236.
16. Richter, D. (1973) *J. Biol. Chem. 248*, 2853–2857.
17. Bermek, E., Krämer, W., Mönkmeyer, H. & Matthaei, H. (1970) *Biochem. Biophys. Res. Commun. 40*, 1311–1318.
18. Bermek, E. & Matthaei, H. (1971) *Biochemistry, 10*, 4906–4912.
19. Modolell, J., Cabrer, B. & Vazquez, D. (1973) *Proc. Natl Acad. Sci. U.S.A. 70*, 3561–3565.
20. Gordon, J. (1969) *J. Biol. Chem. 244*, 5680–5686.
21. Skoultchi, A., Ono, Y., Waterson, J. & Lengyel, P. (1970) *Biochemistry, 9*, 508–514.
22. Hardesty, B., Culp, W. & McKeehan, W. (1969) *Cold Spring Harbor Symp. Quant. Biol. 34*, 331–345.
23. Traugh, J. A. & Collier, R. J. (1971) *FEBS Lett. 14*, 285–288.
24. Smulson, M. E. & Rideau, C. (1970) *J. Biol. Chem. 245*, 5350–5353.
25. Rao, P. & Moldave, K. (1969) *J. Mol. Biol. 46*, 447–457.
26. Watanabe, S. (1970) *J. Antibiot. (Tokyo) Ser. A, 23*, 313–314.

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