DISSOCIATION OF AMINOACYL tRNA FROM THE COMPLEX OF EF-Tu--GTP--AMINOACYL tRNA BY EXTRACTS OF *ESCHERICHIA COLI*

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SUMMARY: Ribosome-free extracts of freshly-grown cells contain a heat-labile, nondialyzable component which causes the dissociation of aminoacyl tRNA from the ternary complex of EF-Tu--GTP--aminoacyl tRNA. This activity does not dissociate EF-Tu--GTP, deacylate aminoacyl tRNA, or degrade GTP, and is not due to EF-Ts.

Escherichia coli contains 3 proteins that are required for the elongation of nascent polypeptides on ribosomes: elongation factors Tu, Ts, and G (1).[1] The concentration of EF-Tu is regulated differently from that of EF-G, EF-Ts, and ribosomes (2). The concentrations of the latter three are directly proportional to growth rate and are maintained in equimolar amounts (3-5). In contrast, while the level of EF-Tu does increase with growth rate, the molar ratio of EF-Tu and ribosomes is considerably greater than 1 and also varies with the growth rate, dropping from a value of about 15 in cells doubling 0.4 times per hour or slower, to about 8 in cells doubling twice per hour (2).

The levels of EF-Tu parallel those found for tRNA by Sköld *et al.* (6). As was pointed out earlier (2), this correspondence of EF-Tu and tRNA fits with the proposed role of EF-Tu in protein synthesis, which is to form a ternary complex with GTP and aminoacyl tRNA, thereby facilitating access of the aminoacyl tRNA to the ribosomal A site, where codon recognition takes place (1). Thus, even if most of the tRNA were aminoacylated, there would be sufficient EF-Tu to bind it. Neidhardt *et al.* (7) recently showed that the concentrations of ten aminoacyl tRNA synthetases are maintained at a constant molar ratio to EF-Tu in cells growing at different rates. They pointed out that the synthesis of all of the

[1] Abbreviations: EF-Tu, EF-Ts, and EF-G, elongation factors Tu, Ts, and G. MOPS, morphilinopropane sulfonic acid.

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components necessary to supply the ribosomal A site with aminoacyl tRNA are apparently regulated in parallel.

In order to examine more closely the relationship between EF-Tu and aminoacyl tRNA, I attempted to isolate complexes of them from cell extracts using methods described by others to isolate the ternary complex of EF-Tu--GTP--aminoacyl tRNA (8). Although I could recover the ternary complex from reactions containing purified EF-Tu, I was unable to do so from reactions containing as the source of EF-Tu unfractionated ribosome-free extracts of freshly-grown cells. I found that these extracts contain a heat-labile, nondialyzable material that causes the dissociation of aminoacyl tRNA from the ternary complex.

MATERIALS AND METHODS

Materials — Cell extracts were prepared from a strain of *E. coli* B, NF314, that was obtained from the collection of Niels Fiil. Cells at a density of approximately 4 x 10^8 cells/ml in L-broth (9) were chilled by cooling the culture in ice-slush, collected by centrifugation, and washed once with buffer A [10 mM Tris-HCl (pH 7.4), 10 mM magnesium acetate, 0.1 M KCl, 20% (vol/vol) glycerol, 10 mM 2-mercaptoethanol, and 0.58 mM phenylmethanesulfonyl fluoride]. The washed cells were suspended in 4 volumes of buffer A and ruptured by passage through a French press; ribosome-free extracts were prepared as described earlier (10). The 3-ml capacity French press (catalog No. 4-3399, American Instrument Co.) was routinely used because ribosome-free extracts prepared by this press reproducibly contain significantly less nuclease than ribosome-free extracts made from similar cells ruptured in the 40-ml capacity press (catalog No. 4-3398). The reason for this difference is not known, but the cells are extruded at a much faster rate from the smaller press.

Purified EF-Tu and partially purified EF-Tu--EF-Ts were prepared as previously described (2, 10). The amount of EF-Tu and of EF-Tu--EF-Ts was determined as described (2). EF-Ts activity was measured as described by Arai *et al.* (11). Using their definition of a unit as the amount of EF-Ts activity which catalyzes the exchange of 1 pmol of GDP from EF-Tu--GDP per minute at 0°, the EF-Tu--EF-Ts used here had 11.5 units per pmol of EF-Ts. This value agrees with the value of 12.3 for pure EF-Ts (11).

The aminoacylation of tRNA by a mixture of 15 [^{14}C]amino acids (New England Nuclear) was carried out as described for the preparation of phenylalanyl tRNA (10). Deacylated [^{3}H]tRNA was isolated by phenol extraction of a ribosome-free extract of cells that had been grown to early logarithmic phase in MOPS medium (12), containing 2 μCi per ml of [^{3}H]uracil (20.4 μCi/nmol).

Methods — The reaction to form the ternary complex between EF-Tu, GTP, and aminoacyl tRNA was carried out following the general procedures of Weissbach *et al.* (13). Unless indicated otherwise, 50-μl solutions containing 80 to 90 pmol of EF-Tu were incubated for 10 minutes at 30° with 0.025 mM [^{3}H]GDP, 2 mM phosphoenolpyruvate, 2.5 μg per ml of pyruvate kinase (rabbit skeletal muscle, Sigma), 0.16 M NH_4Cl, 5 mM magnesium acetate, 10 mM 2-mercaptoethanol, and 50 mM Tris-HCl, pH 7.5. In reactions containing both purified EF-Tu and the EF-Tu in the ribosome-free extract, 80 to 90 pmol of each were present. 0.11 of an

$A_{260\ nm}$ unit of [^{14}C]aminoacyl tRNA (5,600 cpm, of which about 80% were precipitable with cold trichloroacetic acid) was added, and after an additional 5 minutes at 30° the reaction was chilled on ice. Any variations of this protocol are given in the legends to the figures.

Portions of the reactions were chromatographed on 1-ml columns (fashioned from a 1-cc tuberculin syringe) packed with Sephadex G-75 superfine equilibrated in the same buffer-salts solution used above but containing no GDP or GTP and one-half the concentration of phosphoenolpyruvate and pyruvate kinase. The column was developed at room temperature at 5.4 ml per hour, and the radioactivity of each 1-drop fraction was determined in a liquid scintillation spectrophotometer. In different experiments 65% to 85% of the EF-Tu--[^{3}H]GTP was recovered. Chromatography on Sephadex columns was used by Ono *et al.* (8) to separate unbound aminoacyl tRNA (or tRNA) from the EF-Tu--GTP--aminoacyl tRNA complex, and the results that I obtained with purified EF-Tu are analogous to theirs. In experiments not presented I found that the formation of the ternary complex was completely dependent on phosphoenolpyruvate and pyruvate kinase, which quantitatively converts to GTP both the GDP in solution and, as Weissbach *et al.* showed (14), the GDP bound to EF-Tu.

RESULTS AND DISCUSSION

Figs. 1a and 1b show that a ternary complex of EF-Tu--[^{3}H]GTP--[^{14}C]aminoacyl tRNA (indicated by the vertical arrows) can be recovered from reaction mixtures containing purified EF-Tu by filtration through Sephadex columns. Fig. 1a shows the correspondence of [^{14}C]aminoacyl tRNA and [^{3}H]GTP in fractions containing the ternary complex, and Fig. 1b shows that the [^{14}C]aminoacyl tRNA bound in the ternary complex is eluted earlier than the [^{3}H]tRNA which was used here as a marker for unbound aminoacyl tRNA. In the reaction shown in Fig. 1b, nonradioactive GTP was used instead of [^{3}H]GTP. In experiments not shown, I found that the chromatographic position of EF-Tu--GDP or of EF-Tu--GTP on the columns used here is essentially the same as that of the ternary complex. A similar result was reported by Ono *et al.* (8).

In contrast to these results, Figs. 1c and 1d show that EF-Tu--[^{3}H]GTP but little or no ternary complex is recovered from reactions using a ribosome-free extract (S100) as the source of EF-Tu. There is a significant decrease in the amount of [^{14}C]aminoacyl tRNA in the fractions that would contain the ternary complex (*cf.*, Figs. 1c and 1a) and essentially all of the [^{14}C]aminoacyl tRNA has been eluted as unbound aminoacyl tRNA (*cf.*, Figs. 1d and 1b).

The inability to recover the ternary complex from reactions using S100 as the source of EF-Tu cannot be explained by a deacylation of aminoacyl tRNA

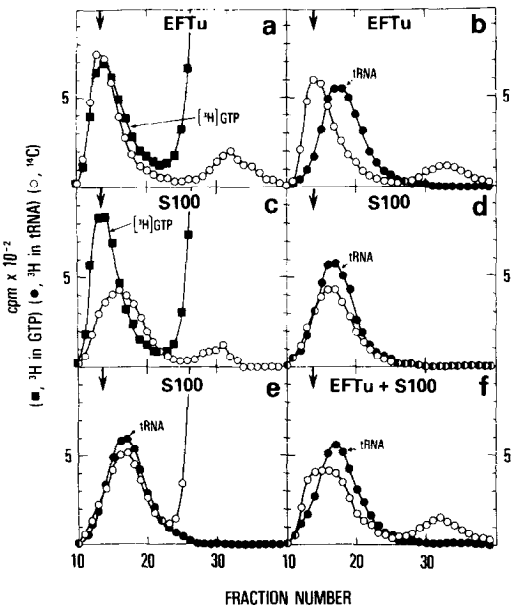

FIG. 1. Recovery of the ternary complex from reactions with different sources of EF-Tu. All the reactions were carried out and analyzed by column chromatography as described in "Materials and Methods," and each reaction contained approximately the same amount of purified EF-Tu or the EF-Tu present in the S100 or a mixture of both. *Panel a* — This reaction contained purified EF-Tu, [^{14}C]aminoacyl tRNA, and [^{3}H]GTP. The vertical arrow indicates the position of the ternary complex, and excess, unbound [^{3}H]GTP is recovered in fractions 25 to 39. About 20% of the ^{14}C radioactivity was recovered in these fractions as free [^{14}C]amino acids, a value which agrees with the determination that about 20% of the ^{14}C radioactivity in the [^{14}C]aminoacyl tRNA used in these experiments was not precipitable by cold trichloroacetic acid (see "Materials and Methods"). In experiments not shown, it was found that the extent of deacylation of the [^{14}C]aminoacyl tRNA as determined by acid precipitation agreed well with the relative recovery of free [^{14}C]amino acids and [^{14}C]aminoacyl tRNA from these columns. *Panel b* — Same as panel *a*, except that [^{3}H]GTP was replaced with GTP and about 5,000 cpm of [^{3}H]tRNA was added just before the chromatography. *Panels c and d* — Same as panels *a* and *b*, respectively, except that the S100 was used as the source of EF-Tu. For the results in panel *d*, the radioactivity of the fractions was precipitated by cold trichloroacetic acid using a method described earlier (16). For this reason, no free [^{14}C]amino acids were recovered in fractions 25 to 39. *Panel e* — The same as panel *d*, except that the exogenous [^{14}C]aminoacyl tRNA was replaced by 1 mM ATP, 2 μCi/ml of a mixture of 15 [^{14}C]amino acids, and an excess of a mixture of aminoacyl tRNA synthetases partially purified as described earlier (10). *Panel f* — Same as panels *b* or *d*, except that both purified EF-Tu and S100 were used.

because the relative recovery of [^{14}C]aminoacyl tRNA from these reactions is the same as that from reactions containing purified EF-Tu (*cf.*, Figs. 1a, 1b, and 1c). In addition, the recovery of [^{14}C]aminoacyl tRNA from the column shown in Fig. 1d, where only the radioactivity precipitable by cold trichloroacetic acid

was determined, was the same as that from the column of Fig. 1c. In order to rule out that the EF-Tu in the S100 might only be able to form a ternary complex with the aminoacyl tRNA synthesized from the tRNA in the S100, I carried out a reaction in which the exogenous [^{14}C]aminoacyl tRNA was replaced with [^{14}C]amino acids, ATP, and excess aminoacyl tRNA synthetases. As Fig. 1e shows, essentially all of the aminoacyl tRNA synthesized from the endogenous tRNA cochromatographed with the [^{3}H]tRNA used as a marker, indicating that little or no ternary complex was recovered.

As a comparison of Figs. 1f and 1b shows, only a small amount of ternary complex is recovered from a reaction containing both purified EF-Tu and S100. This same result was found if the S100 was added after the ternary complex with purified EF-Tu was formed. This finding, which is illustrated below, suggests that something in the S100 affects the stability of the ternary complex. Although the component of the S100 responsible for this effect has not yet been identified, those possibilities that one can apprehend from the known reactions of EF-Tu (1) have been ruled out. Those pertinent to the present discussion are:

$$\text{EF-Tu--EF-Ts} + \text{GTP} \rightleftarrows \text{EF-Tu--GTP} + \text{EF-Ts} \qquad \text{(I)}$$

$$\text{EF-Tu--GTP} + \text{aminoacyl tRNA} \rightleftarrows \underset{\text{(ternary complex)}}{\text{EF-Tu--GTP--aminoacyl tRNA}} \qquad \text{(II)}$$

Anything that reduces the amount of EF-Tu--GTP or of [^{14}C]aminoacyl tRNA could result in a loss of the ternary complex. The results shown in Fig. 1 revealed that the added aminoacyl tRNA was not deacylated, but three possibilities remain:

(i) EF-Ts in the S100 binds to EF-Tu, thereby reducing the amount of EF-Tu--GTP. This explanation is most unlikely. First, because the amount of EF-Ts in the S100 would only be 10% to 15% of the amount of EF-Tu (2, 10), and second, because GTP has been found by Miller and Weissbach (15) to dissociate EF-Tu--EF-Ts. Finally, as Fig. 2a shows, addition of EF-Ts to a reaction containing purified EF-Tu does not reduce the yield of ternary complex. Adding 6 times as much EF-Ts as was used here also had no effect (data not shown).

(ii) A very active GTPase in the S100 destroys the GTP and hence the EF-Tu--

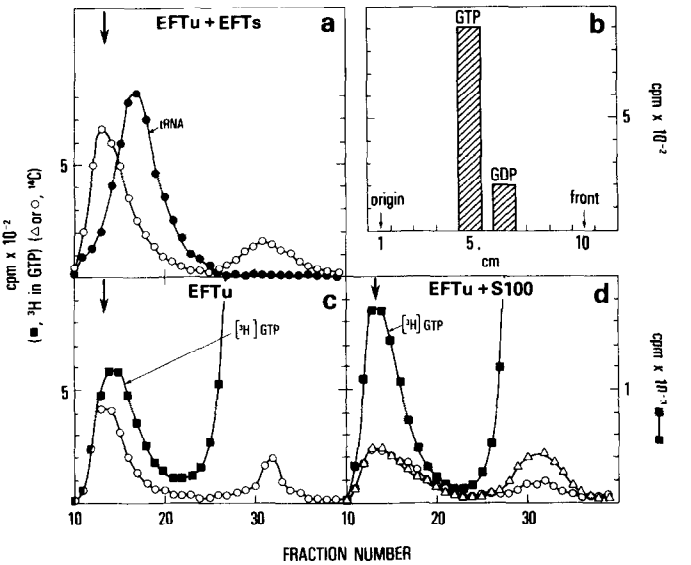

FIG. 2. Control experiments: *Panel a: effect of EF-Ts on the recovery of the ternary complex.* The ternary complex was formed using purified EF-Tu and 8 pmol of EF-Tu--EF-Ts which provided 92 units of EF-Ts activity. *Panel b: analysis of the guanine nucleotide bound to EF-Tu.* A reaction mixture containing purified EF-Tu, S100, [^{14}C]aminoacyl tRNA, and [^{3}H]GTP was incubated and then chromatographed, producing results similar to those shown in Fig. 2d. A portion of the EF-Tu--[^{3}H]GTP peak was made to 1 M HCOOH, and after 10 minutes at 0°, centrifuged. Some of the supernatant was chromatographed on polyethyleneimine cellulose in 1.5 M KH_2PO_4. The radioactivity of the chromatogram corresponding to GDP and GTP, and of 1-cm segments of the remaining portions, including the part located between GDP and GTP, was determined. *Panels c and d: effect of incubation of the S100 on its ability to cause dissociation of the ternary complex.* Separate portions (0.03 ml, 35 pmol EF-Tu) of a reaction mixture containing the ternary complex prepared from purified EF-Tu were incubated for 5 minutes with one of the following: buffer A, S100 (110 pmol EF-Tu) or S100 which had been first incubated alone at 37° for 85 minutes to deacylate any aminoacyl tRNA. This period of time was sufficient to deacylate 97% of a small amount of [^{14}C]aminoacyl tRNA that had been added to the S100. Panel *c* shows the results of the the control experiment, and panel *d* shows the superimposed results of the two reactions that contained S100. The open triangles represent the ^{14}C radioactivity when the ternary complex was reacted with the incubated S100; part of the ^{14}C radioactivity found in the fractions containing free amino acids is derived from the hydrolyzed [^{14}C]aminoacyl tRNA that was added to this S100 to monitor the extent of deacylation. The open circles represent the ^{14}C radioactivity when the ternary complex was reacted with the unincubated S100. The elution of EF-Tu--[^{3}H]GTP was similar for both of the experiments shown in panel *d*, and thus only the results of one of these is shown. Note that the ^{3}H scale, but not the ^{14}C scale, in panel *d* is twice that in panel *c*.

GTP complex. This possibility was ruled out by analyzing the [^{3}H]nucleotide bound to EF-Tu isolated by column chromatography of a reaction mixture containing [^{3}H]GTP, purified EF-Tu, S100, and the rest of the components required to

make the ternary complex. The results of this chromatography resembled those shown in Fig. 2d. The [^{3}H]nucleotide was isolated from a portion of the EF-Tu--[^{3}H]nucleotide peak. As Fig. 2b shows, 80% of the recovered radioactivity was in the form of [^{3}H]GTP.

(iii) Endogenous nonradioactive aminoacyl tRNA in the S100 diluted the exogenous [^{14}C]aminoacyl tRNA. This possibility was eliminated by an experiment which showed that S100, after a 90-minute incubation at 37° which deacylated 97% of a small amount of added [^{14}C]aminoacyl tRNA, was just as efficient in reducing the yield of a ternary complex formed with purified EF-Tu as an S100 that had not been "preincubated." For this experiment a ternary complex was formed with purified EF-Tu, [^{3}H]GTP, and [^{14}C]aminoacyl tRNA. Separate portions of the reaction mixture were then treated with buffer A (Fig. 2c) or with either the incubated or unincubated S100 (Fig. 2d). Because the EF-Tu in the added S100 can bind [^{3}H]GTP, significantly more EF-Tu--[^{3}H]GTP was recovered from the columns shown in Fig. 2d than from that shown in Fig. 2c. However, significantly less ternary complex was recovered from the reactions to which either S100 was added than from the reaction which contained only purified EF-Tu. Furthermore, the experiments in Fig. 2d show that adding the S100 after the ternary complex was formed was just as effective in reducing the yield of the complex as adding the S100 before the complex was formed (*cf.*, the elution profile of [^{14}C]aminoacyl tRNA in Figs. 2d and 1f).

By use of the column assay, I have found that the component in the S100 responsible for the poor recovery of the ternary complex is heat-labile and nondialyzable (data not shown). I also have found that very little, if any, of it is present in cells that have been stored at -70° for an extended period of time (5 years or more), even though such extracts contain EF-Tu of the same specific activity as that in freshly-grown cells. On the other hand, the activity of the component is stable for at least 6 weeks at 4° in the ribosome-free extracts of freshly-grown cells. The means by which this activity dissociates aminoacyl tRNA from the ternary complex and the possible role of such an activity in protein synthesis is currently being investigated.

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