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Elongation Factor Tu Ternary Complex Binds to Small Ribosomal Subunits in a Functionally Active State†

Jerome A. Langer,‡ Frances Jurnak, and James A. Lake*

ABSTRACT: A complex between elongation factor Tu (EF-Tu), GTP, phenylalanyl-tRNA (Phe-tRNA), oligo(uridylic acid) [oligo(U)], and the 30S ribosomal subunit of *Escherichia coli* has been formed and isolated. Binding of the EF-Tu complex appears to be at the functionally active 30S site, by all biochemical criteria that were examined. The complex can be isolated with 0.25–0.5 copy of EF-Tu bound per ribosome. The binding is dependent upon the presence of both the aminoacyl-tRNA and the cognate messenger RNA. Addition of 50S subunits to the preformed 30S–EF-Tu–GTP–Phe-tRNA–oligo(U) complex ("30S–EF-Tu complex") causes a rapid hydrolysis of GTP. This hydrolysis is coordinated with the formation of 70S ribosomes and the release of EF-Tu. Both the release of EF-Tu and the hydrolysis of GTP are stoichiometric with the amount of added 50S subunits. 70S ribosomes, in contrast to 50S subunits, neither release EF-Tu nor rapidly hydrolyze GTP when added to the 30S–EF-Tu complexes. The inability of 70S ribosomes to react with the 30S–EF-Tu complex argues that the 30S–EF-Tu complex does not dissociate prior to reaction with the 50S subunit. The requirements of the 30S reaction for Phe-tRNA and oligo(U) and the consequences of the addition of 50S subunits resemble the reaction of EF-Tu with 70S ribosomes, although EF-Tu binding to isolated 30S subunits does not occur during the elongation microcycle. This suggests that the EF-Tu ternary complex binds to isolated 30S subunits at the same 30S site that is occupied during ternary complex interaction with the 70S ribosome. These data also suggest that crucial parts of the 70S binding site for the EF-Tu complex may be on the 30S ribosomal subunit in locations where they do not significantly interfere with subunit association. Because this 30S–EF-Tu complex can be isolated from sucrose gradients, three-dimensional immune mapping of the EF-Tu binding site directly on 30S subunits is feasible.

During protein synthesis, a ternary complex of elongation factor Tu, aminoacyl-tRNA (aa-tRNA),[1] and GTP binds to the 70S ribosome. The binding of this complex is the initial step for the translation of a particular codon [for reviews, see Lengyel (1974), Kaziro (1978), and Lake (1981)]. Immediately following binding of the complex containing the correct aminoacyl-tRNA, GTP hydrolysis occurs, EF-Tu is released, and peptidyl transfer occurs. Hence the binding of the complex to its initial site on the 70S ribosome, the recognition or R site (Lake, 1977; Johnson et al., 1977), is transient. In this paper we show that if only 30S subunits are present, it is possible to obtain stable binding of the EF-Tu complex. Unlike a similar 30S–EF-Tu complex previously reported and characterized (Brot et al., 1970; Weissbach et al., 1972a), GTP is rapidly hydrolyzed when 50S subunits are added to this 30S–EF-Tu complex. In other respects as well, the functional properties of our 30S–EF-Tu complex are like those of the interaction of the complex with 70S ribosomes. Although the 30S–EF-Tu complex plays no role in the elongation microcycle

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[1] Abbreviations: aa-tRNA, aminoacyl-tRNA; EF-Tu, elongation factor Tu; GMPPCP, guanosine 5′-(β,γ-methylenetriphosphate); oligo(U), oligo(uridylic acid); Phe-tRNA, phenylalanyl-tRNA; Tris, tris(hydroxymethyl)aminomethane.

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of protein synthesis, these results strongly suggest that the EF-Tu complex is bound to the same site on the 30S subunit in both the 30S and 70S complexes. We anticipate that the 30S–EF-Tu complex will be useful in functional studies on elements contributing to the binding and release of EF-Tu, as illustrated here by experiments on the role of L7/L12, and in structural studies, e.g., immune electron microscopy to localize the recognition site.

Experimental Procedures

Biochemical Materials. Ribosomal subunits were prepared from tight couples of MRE600 ribosomes (Hapke & Noll, 1976) on sucrose gradients (Staehelin et al., 1969; Eikenberry et al., 1970). 30S and 50S subunit preparations had less than 5% molar cross-contamination, as judged by optical density on analytical sucrose gradients. The fast-sedimenting shoulder seen in the 30S preparation (Figure 2A) mostly appears after incubation of 30S ribosomal subunits with oligo(U) and the EF-Tu ternary complex.

50S subunits lacking proteins L7/L12 were prepared by twice extracting 50S subunits with 0.5 M NH_4Cl–50% ethanol at 0 or 37 °C, producing particles referred to as "50S P_0" or "50S P_{37}", respectively (Hamel et al., 1972). The efficiency of extraction was checked on agarose/acrylamide gels (Tokimatsu et al., 1981), from which the presence of 50S subunits retaining L7/L12 was estimated at less than 5%. 50S-P_0 particles were tested in a poly(U)-directed polyphenylalanine synthesis assay (Gesteland, 1966) and were found to have about 20% of the activity of unextracted 50S subunits, in agreement with previous investigators (Weissbach et al., 1972b; Strycharz et al., 1978). In these assays the incorporation of phenylalanine in reactions containing unmodified 50S subunits was about 60 phenylalanines per ribosome (100%) in a 40-min incubation at 37 °C. The addition of extracted proteins (SP_0) to the 50S-P_0 reactions led to recovery of significantly greater than 100% activity. This enhancement of activity has been reported previously (Hamel et al., 1972; Weissbach et al., 1972b) and may be related to a partial depletion of L7/L12 from unextracted 50S subunits during their preparation (Cohlberg, 1980); indeed, Brot et al. (1973) have described the binding of a small amount of [^{3}H]L7/L12 to 50S subunits.

Phenylalanyl-tRNA was prepared from crude tRNA (from *Escherichia coli* MRE600; Boehringer/Mannheim) by acylation (Ravel & Shorey, 1971) and subsequent purification on BD-cellulose (Bio-Rad; Lucas-Lenard & Haenni, 1969). It was extensively dialyzed against 10 mM ammonium acetate and 0.5 mM magnesium acetate, lyophilized in small aliquots, and stored at −80 °C. The separation of Phe-tRNA from unacylated tRNAs on BD-cellulose is very efficient, allowing a specific activity of 1100 pmol/A_{260} unit (Boehringer/Mannheim, 1984/1985) to be used. Since Phe-tRNA is used in considerable excess, deviations from this value are unlikely to have significant effects. EF-Tu–GDP was prepared as described elsewhere (Louie et al., 1984), and the EF-Tu–GDP was phosphorylated with pyruvate kinase and phosphoenolpyruvate in the presence of 0.1 mM GTP and 2.94 μM [8-^{3}H]GTP (specific activity 17 Ci/mmol; ICN). The resulting EF-Tu–GTP was stored in the presence of the phosphorylating system at −80 °C. Antibiotic X-5108 (aurodox), the *N*-methyl form of kirromycin (Maehr et al., 1973), was the gift of Dr. Hubert Maehr (Hoffmann-La Roche, Inc., Nutley, NJ). The antibiotic was made up as a 2–3 mM solution in 20% ethanol and was used at a final concentration of 20–40 μM.

Calculation of ribosome stoichiometries was on the basis of the extinction coefficients and molecular weights of Hill et al. (1969), which convert to 1 A_{260} unit being equivalent to 76 pmol of 30S subunits, 43 pmol of 50S subunits, or 25 pmol of 70S ribosomes.

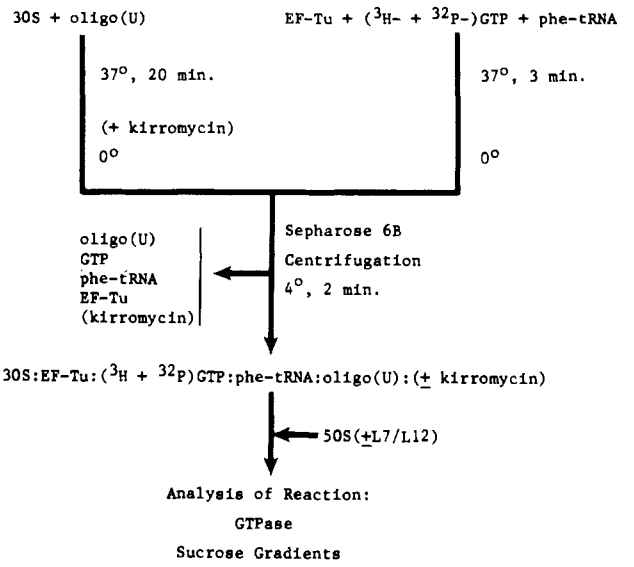

FIGURE 1: Protocol for experiments on the addition of 50S subunits to the 30S–EF-Tu complex. Several possible variations in the experiments are indicated, including use of kirromycin in the formation of the 30S–EF-Tu complex and the possibility of using 50S subunits lacking proteins L7/L12. In the experiments illustrated in this paper, 50S subunits contained proteins L7/L12.

Formation of EF-Tu Complexes. The general protocol for the formation, purification, and analysis of complexes of EF-Tu and the 30S ribosomal subunit is summarized in Figure 1. Complex formation was done with limiting ribosomes to maximize occupancy of ribosomes by EF-Tu. Two mixtures of 100 μL each were made: (1) EF-Tu–GTP (ca. 400 pmol), with GTP having a mixture of [^{1}H]- and [^{3}H]GTP, supplemented with additional [^{3}H]GTP (17 Ci/mmol; ICN) or [γ-^{32}P]GTP and Phe-tRNA (ca. 750 pmol) and (2) 30S subunits (200 pmol = 2.7 A_{260} units) and oligo(U) (45 μg or about 9 nmol of oligomer; Calbiochem). The first was incubated for 3 min at 37 °C, while the second was activated for 20 min at 37 °C. After both mixtures were cooled to 0 °C, they were mixed for a total volume of 200 μL in a buffer that contained approximately 20 mM Tris-HCl, 12 mM magnesium acetate, 75 mM KCl, 75 mM NH_4Cl, and 1 mM dithiothreitol, adjusted to pH 7.6.

Purification of Ribosome–EF-Tu Complexes. Following incubation of the components at 0 °C for 20 min as described above, the 30S–oligo(U)–EF-Tu–GTP–Phe-tRNA complex ("30S–EF-Tu complex") was separated from excess EF-Tu, Phe-tRNA, and small molecules by "centrifugational chromatography". In this procedure, based on the "microcentrifuge desalting" procedures of Penefsky (1977) and Helmerhorst & Stokes (1980), the reaction mixture was layered onto a 1-mL column of Sepharose 6B (in a disposable 1-mL tuberculin syringe fitted with a disk of porous polyethylene to support the Sepharose gel) and was centrifuged at about 1400*g* for 2 min at 4 °C. This column effectively separated EF-Tu complex bound to ribosomes from EF-Tu–GTP–Phe-tRNA (see Figure 2). Free GTP, oligo(U), Phe-tRNA, and *N*-methylkirromycin (when used) were quantitatively retained on the column. Recovery of ribosomes is about 40% of input, or about 80 pmol in 100–150 μL.

Analysis of Complexes on Sucrose Gradients. Complexes were further analyzed on 5-mL 7–25% sucrose gradients having the following ionic composition: 20 mM Tris-HCl, 12 mM magnesium acetate, 75 mM KCl, and 75 mM NH_4Cl,

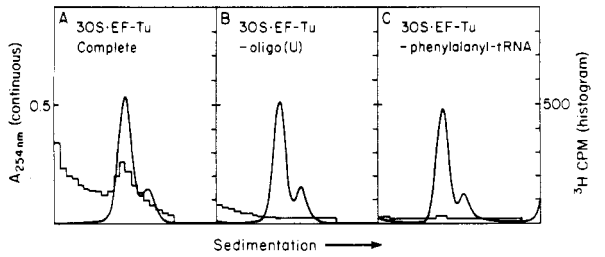

FIGURE 2: EF-Tu binds to the 30S ribosomal subunit in a mRNA-dependent and phenylalanyl-tRNA-dependent manner. Small ribosomal subunits, oligo(U), Phe-tRNA, and [^{1}H]- and [^{3}H]GTP were reacted as described under Experimental Procedures. Unbound components were removed by centrifugation through a 1-mL column of Sepharose 6B. The samples were layered on sucrose gradients and analyzed as described. (A) Complete reaction; (B) complete reaction with oligo(U) omitted; (C) complete reaction with Phe-tRNA omitted. Radioactivity at the top of the gradients represents GTP that was bound to 30S subunits during purification on Sepharose 6B but that subsequently dissociated. The dissociated GTP is probably present as the complex EF-Tu–GTP–Phe-tRNA.

adjusted to pH 7.6. Gradients were spun at 4 °C in an VTi 65 rotor (Beckman) for 40 min at 35 000 rpm or for 20 min at 49 000 rpm and were fractionated with continuous monitoring of absorbance at 254 nm. Fractions of 0.187 mL were collected, diluted with 200 μL of water, and supplemented with 3 mL of scintillation fluid 3a70B (Research Products International Corp.) for determination of ^{3}H and ^{32}P. Counting efficiencies were about 7% for ^{3}H and 51% for ^{32}P.

GTP Hydrolysis. 30S–EF-Tu complexes containing mixtures of [^{1}H]-, [8-^{3}H]-, and/or [γ-^{32}P]GTP were prepared, purified by centrifugational chromatography (as described above), and cooled to 0 °C. 50S subunits [or 70S ribosomes preincubated with oligo(U)] were added to the purified complexes, and GTP hydrolysis was measured by the liberation of P_i from [γ-^{32}P]GTP (Campuzano & Modolell, 1980). At various times after addition of 50S subunits or 70S ribosomes, 10 or 15 μL of the reaction was pipetted into 150 μL of 0.7 M $HClO_4$/2.5 mM KH_2PO_4 with 4% (w/v) activated charcoal (which absorbs nucleotide). After mixing, the charcoal was pelleted in a microfuge (Beckman) for 2 min. A total of 100 μL of the supernatant was added to 2 mL of scintillation fluid 3a70B (Research Products International Corp.) and was counted in a scintillation counter. The presence of [8-^{3}H]GTP in this reaction served as an internal control for the adsorption of nucleotide by the charcoal. The total amount of ^{32}P in a sample was measured by diluting 10 or 15 μL (depending on the experiment) of each reaction into 150 μL of $HClO_4$/KH_2PO_4 lacking charcoal and measuring the ^{32}P of 100 μL of this sample.

Results

In the absence of large ribosomal subunits, binding of EF-Tu (in the presence of aa-tRNA and GTP) to 30S ribosomal subunits can be measured. This binding appears to be physiological since it is strictly dependent upon the presence of both a messenger RNA and the cognate aminoacyl-tRNA. The occurrence of the 30S–EF-Tu complex is illustrated in Figure 2 by the cosedimentation of [^{3}H]GTP with the small ribosomal subunits on sucrose gradients. Since this 30S–EF-Tu complex had been initially purified by centrifugation through a small column of Sepharose 6B (see Experimental Procedures), ^{3}H not bound to the ribosome had already been removed from the sample. The cosedimentation of [^{32}P]GTP (data not shown) also paralleled the ^{3}H distribution. When this complex is analyzed by sucrose gradient centrifugation, approximately 50% of the EF-Tu (represented by the ^{3}H present in the 30S peak, Figure 2A) remains attached to the 30S subunits. About half of the EF-Tu, although initially bound, dissociates during the centrifugation and is present at the top of the gradient (Figure 2A).

EF-Tu binding to 30S subunits depends upon the presence of both messenger RNA and the cognate aminoacyl-tRNA (parts B and C of Figure 2). In the absence of oligo(U), no ^{3}H cosediments with the 30S subunits. However, a small amount of ^{3}H, amounting to about 8% of that found in the presence of oligo(U), is present at the top of the gradient. In the absence of Phe-tRNA (Figure 2C), again no binding of the EF-Tu–GTP is observed (the nonspecific binding is about 1% of the specific binding). In the complete reaction (Figure 2A), using conditions of limiting ribosomes, one obtains an occupancy of 0.25–0.5 EF-Tu per ribosome (calculated as [^{3}H]GTP per ribosome), depending on the preparation and age of the EF-Tu–GTP.

Since EF-Tu binding to 30S subunits is dependent on the presence of both mRNA and a cognate aminoacyl-tRNA, this suggested that the complex might represent functionally significant binding. In order to investigate and test this possibility, we examined the properties of the complex to compare them with details of the EF-Tu–70S ribosome interaction. Because addition of the ternary complex to 70S ribosomes leads to the dissociation of EF-Tu–GDP and to the hydrolysis of GTP, we studied the effects of adding 50S subunits to preformed 30S–EF-Tu complexes.

The addition of 50S subunits to preformed 30S–EF-Tu complexes results in the rapid hydrolysis of GTP. The time course of [^{32}P]P_i liberation is shown in Figure 3A. Within the first 30 s of the addition of 50S subunits (the first time point) at 0 °C, bound GTP is hydrolyzed. This rapid phase is followed by a much slower second step.

70S ribosomes preincubated with oligo(U), when added in stoichiometric amounts to preformed 30S–EF-Tu complexes, are unable to produce the rapid step of hydrolysis (Figure 4). They do, however, produce the slow phase. Since 70S ribosomes cannot release EF-Tu from 30S subunits (see Figure 5F), the slow step of GTP hydrolysis is not associated with EF-Tu release.

During the fast step, the amount of GTP hydrolyzed is proportional to the amount of 50S subunits added. In Figure 3B the release of γ-^{32}P from 30S subunits is plotted as a function of the amount of 50S subunits added. Also shown (open circles) are data on the release of the EF-Tu–GDP from the 30S subunits (measured as the release of [^{3}H]GTP in the experiment to be described in Figure 5). Both experimental results are similar with respect to the shapes of the curves as a function of 50S subunits added. Although the stoichiometries of P_i release and EF-Tu–GDP release are similar, it should be noted that these results come from very different types of experiments. As will be shown more reliably by double labeling in sucrose gradient experiments (Figure 5), P_i release and EF-Tu release are both stoichiometric with added 50S subunits. This suggests that we are measuring two aspects of a coupled reaction. Hence the preformed 30S–EF-Tu complexes are competent to undergo rapid GTP hydrolysis upon the addition of stoichiometric amounts of 50S subunits.

The second effect of adding 50S subunits to preformed 30S–EF-Tu complexes is to promote the rapid release of EF-Tu and the conversion of 30S subunits to 70S monosomes. In Figure 5, the effects of adding increasing amounts of 50S subunits to preformed 30S–EF-Tu complexes are analyzed by sucrose gradient centrifugation. A mixture of [^{3}H]- and [γ-^{32}P]GTP was used to identify the bound nucleotide following initial purification of the complex by centrifugational chro-

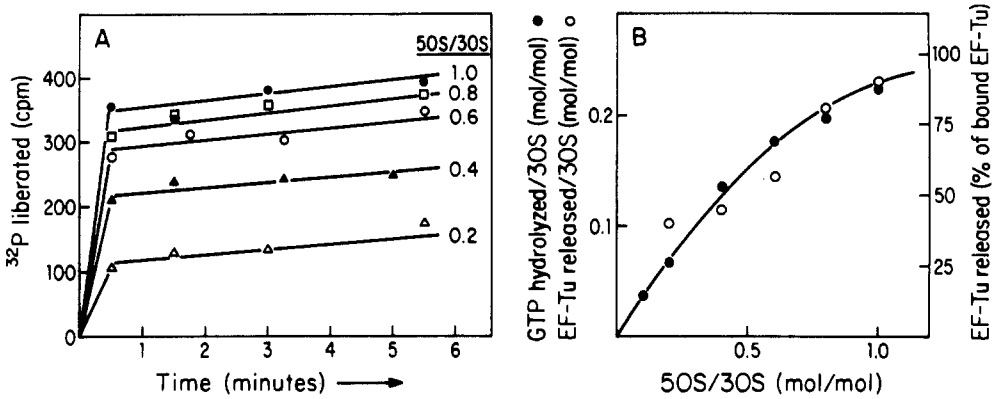

FIGURE 3: Hydrolysis of GTP from 30S–EF-Tu complexes is proportional to the amount of 50S subunits added. (A) 30S–EF-Tu complexes were prepared with [γ-^{32}P]GTP and [^{3}H]GTP, purified as described under Experimental Procedures, and divided into several portions. Different amounts of 50S subunits were added to each aliquot, and the time course of GTP hydrolysis was measured for each reaction. The molar ratios of 50S to 30S subunits are indicated along each curve. For each condition, the amount of [^{32}P]P_i liberated during the first 30 s is plotted, corrected for the hydrolysis in the absence of 50S subunits (86 cpm). At the highest concentration of 50S subunits used, the [^{32}P]P_i liberated in the first 30 s represents hydrolysis of 31% of the total [γ-^{32}P]GTP in the reaction. The straight lines are best fits to the data including 10-min time points that are not shown. (B) Moles of GTP hydrolyzed (●) or of EF-Tu released (○) per mole of 30S subunits is shown as a function of the amount of added 50S subunits. The data for GTP hydrolysis are taken from the experiments in (A), and those for EF-Tu are taken from Figure 5 (see the legend to Figure 5 describing their calculation).

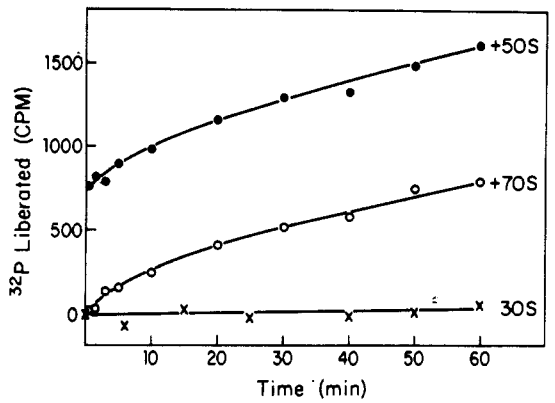

FIGURE 4: 70S ribosomes stimulate the slow phase of GTP hydrolysis of the 30S–EF-Tu complex but not the fast phase. The protocol is as described under Experimental Procedures, but approximately 0.5 mol of 50S subunits or 70S ribosomes was used per mole of 30S subunits. In this experiment, 24% of the total GTP in the reaction was hydrolyzed by 50S subunits in the first 30 s and 50% by the end of 60 min.

matography. In these experiments the release of EF-Tu is found to be proportional to the amount of 50S subunits added. Hence release, by this criterion, does not involve recycling of 50S subunits.

An additional observation is that EF-Tu–GDP release and GTP hydrolysis are coupled in these experiments and that both are stoichiometric with the amount of added 50S subunits. As shown in Figure 5A–E, EF-Tu–GDP release and GTP hydrolysis (measured as the decrease of [^{3}H]GTP and [γ-^{32}P]-GTP, respectively, under the 30S peak) are coupled and are directly proportional to the added 50S subunits. Thus release of both is essentially complete at a 50S/30S (mol/mol) stoichiometry of 1. An additional observation is that the ratio of bound EF-Tu to 30S subunits does not detectably change as a function of the amount of 50S subunits added. This suggests that 50S subunits bind randomly to 30S subunits with a bound EF-Tu complex and subunits lacking EF-Tu.

In order to ascertain if the interaction of the 30S–EF-Tu complex with the 50S subunits is direct or if it first involves the dissociation of the 30S–EF-Tu complex, we added 70S ribosomes preincubated with oligo(U), rather than 50S subunits, to the prebound 30S–EF-Tu complex. As shown by dashed lines in Figure 5F, addition of 70S ribosomes does not hydrolyze [γ-^{32}P]GTP and does not release EF-Tu (data not shown) from the 30S–EF-Tu complex. This experiment suggests that the release is direct, i.e., that it does not involve a prior dissociation of the EF-Tu complex and its subsequent interaction with the 70S ribosome.

In order to demonstrate directly the coupling between these two reactions, we have used an antibiotic known to uncouple GTP hydrolysis from EF-Tu binding. This allowed us, in one experiment, to measure the transfer of EF-Tu to the 70S ribosome and the hydrolysis of GTP. The antibiotic *N*-methylkirromycin stabilizes the EF-Tu–70S complex, while permitting the hydrolysis of GTP (Wolf et al., 1974). In our experiments (see Figure 6) *N*-methylkirromycin was added to the 30S–EF-Tu reaction mixture prior to purification. Following centrifugational chromatography to eliminate excess reactants, including unbound *N*-methylkirromycin, 50S subunits were added to the 30S–EF-Tu (*N*-methylkirromycin) complex and analyzed on sucrose gradients (Figure 6C). As in the absence of kirromycin (Figure 6B), the 50S subunits cause a large decrease in the 30S peak with its bound EF-Tu; however, the majority of EF-Tu is now found in the 70S peak. The EF-Tu is now complexed with GDP, as shown by the cosedimentation of ^{3}H (but not ^{32}P) with the 70S peak. The hydrolysis of GTP has been nearly 100%, as indicated by the absence of ^{32}P under the 70S peak, and the liberated ^{32}P is now present at the top of the gradient (Figure 6C). Hence we conclude that transfer of EF-Tu to the 70S ribosome from the 30S–EF-Tu complex has been coupled with a 1/1 stoichiometry to the hydrolysis of the γ-phosphate of GTP.

Experiments similar to those described above have been performed with 50S subunits lacking proteins L7/L12. The addition of 50S-P_0 particles to the 30S–EF-Tu complex causes the release of EF-Tu from the 30S subunit, with the consequent formation of a 30S–50S-P_0 ("70S-P_0") particle having no bound nucleotide, with the extent of the release proportional to the amount of 50S-P_0 particles added (the data are essentially identical with those presented in Figure 5). Similarly, when *N*-methylkirromycin was included in the original incubation with 30S subunits and EF-Tu, the addition of 50S-P_0 particles to the purified 30S–EF-Tu (+kirromycin) complex produced a 70S-P_0 peak having bound EF-Tu and GDP, similar to that seen with native 50S subunits (Figure 6C). Finally, the addition of 50S-P_0 or 50S-P_{37} particles to the 30S–EF-Tu

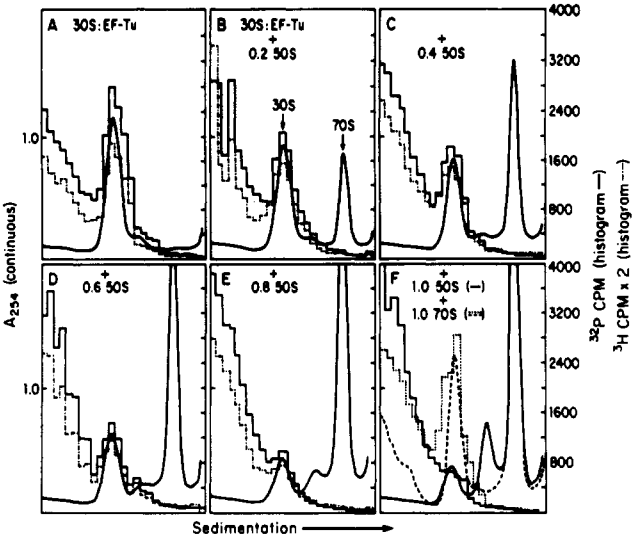

FIGURE 5: The release of EF-Tu from preformed 30S complexes is proportional to the amount of 50S subunits added, and 70S ribosomes are unable to release EF-Tu. The protocol is described under Experimental Procedures. Fractional amounts of 50S subunits were added to 0.63 A_{260} unit of 30S–EF-Tu complexes (ca. 46 pmol of 30S subunits) in 150 μL. Reactions were analyzed on sucrose gradients. Centrifugation was begun within 20 min of adding the 50S subunits and was continued for 20 min. Both ^{32}P (—) and ^{3}H (–·–) are shown. Optical density at the top of the gradient in (F) is from the excess oligo(U) that was added with 70S ribosomes. In this sample the free oligo(U) was not removed by centrifugational chromatography prior to analysis on the sucrose gradient. The ratios are (A) no 50S subunits, (B) 0.2 50S subunit per 30S subunit or 0.22 A_{260} unit (corresponding to about 9.6 pmol) of 50S, (C) 0.4 50S subunit per 30S subunit, (D) 0.6 50S subunit per 30S subunit, (E) 0.8 50S subunit per 30S subunit, and (F) 1.0 50S subunit per 30S subunit. The dashed lines in (F) correspond to the addition of an equimolar amount of 70S ribosomes (1.9 A_{260} units). The ^{3}H data, corresponding to the bound ternary complex, are plotted in summary form in Figure 3B (open circles) by the following calculations. To quantitate the conversion of 30S subunits to 70S ribosomes, the 30S peaks were integrated. Occupancy of EF-Tu was calculated by summing the ^{3}H cosedimenting with the 30S peak, after subtracting from each gradient the counts present in the minus oligo(U) control (see, e.g., Figure 2B). Because results from two different types of experiments are being compared in Figure 3B, no further corrections (such as those for loss of ternary complex during centrifugation, for EF-Tu–GTP release during loading the gradients, etc.) were made. Following centrifugation, complexes on the sucrose gradients had an occupancy of 0.53–0.59 GTP (EF-Tu) per 30S subunit. The 100% EF-Tu release was calculated on the assumption of a smooth background curve.

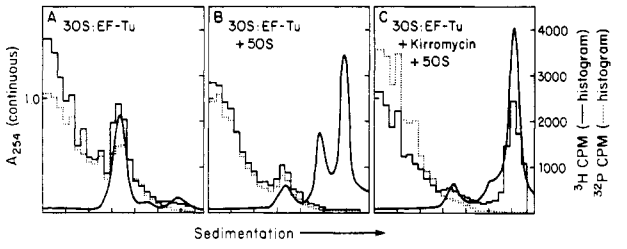

FIGURE 6: Kirromycin prevents the 50S-mediated release of EF-Tu from preformed 30S–EF-Tu complexes, while allowing the 50S-mediated hydrolysis of GTP. In these experiments the release of the γ-phosphate of GTP from the 70S peak was complete. 30S–EF-Tu complexes were prepared in the absence (B) or the presence (C) of *N*-methylkirromycin. Approximately stoichiometric amounts of 50S subunits were added to each sample, and the reaction was analyzed on sucrose gradients. (A) 30S–EF-Tu complex; (B) 30S–EF-Tu complex plus 50S subunits; (C) 30S–EF-Tu complex made with *N*-methylkirromycin plus 50S subunits.

complex at 0 °C led to the rapid hydrolysis of GTP, followed by a continued slow hydrolysis. These results were similar to those for 50S subunits (Figure 4) with the amount of GTP rapidly hydrolyzed being proportional to the amount of 50S-P_0 or 50S-P_{37} particles added. However, about 30% more 50S-P_0 particles than 50S subunits were required, presumably due to the presence of damaged particles in the 50S-P_0 preparation. Nevertheless, since hydrolysis of GTP is stoichiometric with 50S subunits, the observed hydrolysis by 50S-P_0 or 50S-P_{37} particles far exceeds the maximum effect that could be caused by contaminating native 50S subunits. Thus, 50S subunits lacking proteins L7/L12 are similar to unmodified 50S subunits in their ability to cause the dissociation of EF-Tu and the hydrolysis of GTP from the preformed 30S–EF-Tu complex.

Table I: Functional Properties of the 30S–EF-Tu Complex Are the Same as Those Observed for the 70S Ribosome[a]

	30S + EF-Tu–GTP–aa-tRNA	70S + EF-Tu–GTP–aa-tRNA
EF-Tu binding is message dependent	+	+
EF-Tu binding is aa-tRNA dependent	+	+
	+50S[b]	
EF-Tu is released after initial binding	+	+
EF-Tu release is proportional to amount of 50S present	+	+
EF-Tu is not released in the presence of kirromycin	+	+
GTP is hydrolyzed after initial binding	+	+
GTP hydrolysis is rapid	+	+
GTP hydrolysis is proportional to amount of 50S present	+	+
GTP is hydrolyzed in the presence of kirromycin	+	+

[a] Properties of the 30S complex as determined in this paper (shown in the column on the left) are compared with those known for the 70S–EF-Tu interaction (right-hand column). [b] Properties measured after adding 50S subunits to the preformed 30S–EF-Tu complex.

Discussion

The results of the previous section characterize the binding of the EF-Tu ternary complex to the 30S subunit. The properties of the binding and release of the ternary complex to 30S subunits that we have examined are identical with those of the binding and release of the complex to the 70S ribosome. This strongly suggests that this binding occurs at the functional site on the 30S subunit.

While our data suggest that binding occurs at the same site of the 30S subunit in both the 30S and 70S reactions, they do not imply that EF-Tu binding to 30S subunits has any natural role in the elongation cycle. EF-Tu binding to 30S subunits, for example, is nonphysiological in at least one aspect since it occurs in the absence of a tRNA bound to the P site. The properties that we have measured for the 30S complex in this paper are listed in Table I and are compared with those that have been previously published for the 70S ribosome interaction.

(*1*) *Formation of the 30S Complex Is Message Dependent.* The binding of EF-Tu to the 30S subunit is clearly shown in Figure 2. We routinely obtain 0.25–0.5 EF-Tu bound per ribosome and, of this, 0.15–0.3 survives the sucrose gradient purification. This binding is similar to binding on 70S ribosomes in its stringent requirement for both oligo(U) and Phe-tRNA (Figure 2B,C). This is a central property for the complex, if it is to be binding at the 30S functional site.

(*2*) *EF-Tu Is Rapidly Hydrolyzed from 30S Complexes When 50S Subunits Are Added.* The most characteristic reactions observed during the interaction of the EF-Tu ternary complex with 70S ribosomes are the release of EF-Tu, the binding of aminoacyl-tRNA, and the hydrolysis of GTP. It

is generally recognized that release of EF-Tu from 70S ribosomes is associated with the immediate or simultaneous activation of guanosinetriphosphatase (GTPase) and hydrolysis of GTP (Haenni & Lucas-Lenard, 1968; Ertel et al., 1968; Lucas-Lenard et al., 1969; Ono et al., 1969; Shorey et al., 1969; Skoultchi et al., 1970; Yokosawa et al., 1973; Spirin, 1978; Kaziro, 1978). The activation of GTPase with consequent release of EF-Tu is thought to come from an interaction of EF-Tu and the 50S subunit (Gordon, 1969). In our experiments, the 30S–EF-Tu complex is indeed stable (Figure 3A) until the addition of 50S subunits, when GTP is hydrolyzed (Figure 3A). We regard the rapid hydrolysis of GTP as a significant feature of our 30S–EF-Tu complex. We find that within the first 30 s of addition of 50S subunits nearly half of the total GTP is hydrolyzed. This is followed by a second, much slower step (ca. 2 h). Since the addition of 70S ribosomes is unable to cause the release of EF-Tu from 30S complexes (Figure 5F) and since the addition of 70S ribosomes only causes the slow step of GTP hydrolysis and not the fast step, we believe this slow phase is not of direct physiological importance and have not characterized it further.

The rapid step is stoichiometric with added 50S subunits (Figure 3B). Both the hydrolysis of GTP during the rapid step and the release of EF-Tu from 30S subunits show a parallel dependence on the concentration of 50S subunits. This suggests that GTP hydrolysis and EF-Tu release correspond to two aspects of a single coupled reaction.

Others have demonstrated the formation of a stable 30S–EF-Tu complex (Brot et al., 1970). They concluded, however, that because it did not hydrolyze GTP in the presence of 50S subunits (Weissbach et al., 1972a), their EF-Tu complex was not bound at the same functional site in both 30S and 70S ribosomes. They did note a 20–25% hydrolysis of bound EF-Tu but considered that this was not "significant hydrolysis (less than 25%) of any ^{32}P that is already bound to the ribosome". Their observed reduction *is entirely consistent with our results* (Figure 3) if one considers that *their experiments were performed at a 50S/30S (mol/mol) ratio of 0.1*. Thus the design of their experiment would be expected to lead to only 10% hydrolysis of the bound GTP. Hence their data are consistent with our results. We disagree, however, with their conclusion since it implicitly assumes that 50S subunits function catalytically in the GTPase reaction, whereas our experiments show that they function stoichiometrically.

(*3*) *EF-Tu Is Released from 30S Complexes When 50S Subunits Are Added.* The experiments illustrated in Figure 5 clearly demonstrate the stoichiometric nature of the release of EF-Tu when 50S subunits are added. Two aspects of the release reaction deserve further comment.

First, the experiments show quite clearly that the interaction of the 30S–EF-Tu complex with the 50S subunit is direct in the sense that it does not involve binding of a free intermediate of EF-Tu–GTP–Phe-tRNA to 70S ribosomes. This is demonstrated by the inability of added 70S ribosomes to hydrolyze GTP or to release EF-Tu from the 30S–EF-Tu complex (shown in Figure 5F). If dissociation of EF-Tu from the 30S complex were obligatory, then 70S ribosomes should produce an effect similar to that found when 50S subunits are added to the 30S–EF-Tu complex. Hence the interaction very likely represents a direct interaction of the 30S–EF-Tu complex with the 50S subunit.

Second, the presence of the prebound EF-Tu ternary complex does not seem to significantly alter the capability of 30S subunits to form 70S couples. This is illustrated by the fractional release experiments of Figure 5. The effect of adding fractional molar equivalents of 50S subunits to preformed 30S–EF-Tu complexes is to fractionally release the EF-Tu. Thus, for example, adding 50S subunits at a 50S/30S ratio of 0.4 will make 70S couples using 40% of the 30S subunits and will release 40% of the bound EF-Tu. Both those 30S subunits with an EF-Tu and those without are competent to form the 70S complex. This can be most easily seen by noting that constant ratios of ^{3}H and ^{32}P to absorbance are found in the unreacted peak. Thus the binding of the EF-Tu complex does not significantly interfere with or enhance subunit association.

(*4*) *GTP Hydrolysis Is Coupled with EF-Tu Release from 30S Complexes When 50S Subunits Are Added.* The antibiotic *N*-methylkirromycin provides a simple method of separating the processes of EF-Tu release and GTP hydrolysis. This antibiotic, when added to 70S ribosomes, separates these two steps by permitting the hydrolysis of GTP but preventing the release of EF-Tu (Wolf et al., 1974, 1977). When 30S–EF-Tu complexes, previously incubated in the presence of kirromycin, are added to 50S subunits, we find that the γ-labeled P_i released to the top of the gradient (Figure 6C) is equal to the EF-Tu–GDP that is transferred to the 70S peak. Thus GTP hydrolysis is seen to be coupled with the binding of the 50S subunit and EF-Tu–GDP release.

A more direct demonstration of the coupling of EF-Tu release and GTP hydrolysis is provided by the double labeling sucrose gradient experiments in Figure 5. In these experiments, both the γ-^{32}P label and the [^{3}H]GDP label, associated with the 30S peak, are released in a coupled manner when 50S subunits are added. According to the criteria of both of these experiments, the processes of EF-Tu release and GTP hydrolysis are coupled when 30S–EF-Tu complexes are added to 50S subunits.

(*5*) *Ribosomal Proteins L7/L12 Are Not Required for the 50S-Mediated Release of EF-Tu and the Coupled Hydrolysis of GTP.* The previous discussion serves to demonstrate that the 30S–EF-Tu complex has a number of properties expected from our knowledge of the interaction of the EF-Tu–GTP–aa-tRNA ternary complex with the 70S ribosome (Table I). However, the purified 30S–EF-Tu complex also serves as a new substrate for investigating the contribution of various factors to the binding of EF-Tu and its release with the associated hydrolysis of GTP. We have thus illustrated the utility of the complex by demonstrating that 50S subunits lacking proteins L7/L12 are fully competent in causing the release of EF-Tu and the rapid hydrolysis of GTP and, in the presence of *N*-methylkirromycin, can cause the hydrolysis of GTP with the subsequent formation of a 70S–EF-Tu(GDP) complex. These results contradict the conventional view that proteins L7/L12 are required for the various GTPase-related reactions of protein synthesis. However, the current system is unique in allowing us to monitor the single turnover of GTP and the coupled release of EF-Tu, in contrast to most previous studies where multiple turnover events were measured. Thus, in retrospect, the significant residual GTPase activity that had been noted in various studies where the role of L7/L12 was examined [e.g., see Hamel & Nakamoto (1972), Hamel et al. (1972), Ballesta & Vazquez (1972), Sander et al. (1972), Sopori & Lengyel (1972), Weissbach et al. (1972b), Koteliansky et al. (1977), and Glick (1977)] may be taken as evidence that proteins L7/L12 are not required for the fundamental interactions and reactions of EF-Tu (and possibly other factors) and the ribosome. While this conclusion was indeed suggested by various authors on the basis of the residual factor-related activity in the absence of proteins L7/L12 [e.g.,

see Hamel & Nakamoto (1972), Ballesta & Vazquez (1972), Weissbach et al. (1972b), and Koteliansky et al. (1977)], the current system, by isolating single turnover reactions, permits a more direct and forceful demonstration of this point. Nevertheless, the fact that proteins L7/L12 strongly influence the ribosomal interactions with EF-Tu and the other factors is well established [reviewed by Moller (1974)], as is the apparent proximity of proteins L7/L12 to the EF-Tu binding site on 70S ribosomes (Highland et al., 1974; Fabian, 1976; San Jose et al., 1976). By limiting the role of proteins L7/L12, our results may help focus attention on other possible roles for proteins L7/L12, such as the kinetics of the reaction (Pettersson & Kurland, 1980; Thompson et al., 1980) or the regulation of tRNA site selection or discrimination. The use of the 30S–EF-Tu complex might permit more direct evaluation of other factors involved in the binding of EF-Tu to the ribosome and in the GTPase-related dissociation of EF-Tu from the ribosome.

Significance of the 30S–EF-Tu Complex. Several groups have reported the binding of the EF-Tu ternary complex to small ribosomal subunits. We have already noted the reports of Brot et al. (1970) and Weissbach et al. (1972a). In addition, in the rat a complex of the 40S ribosomal subunit with EF-1 (the eukaryotic equivalent of EF-Tu) has been reported (Rao & Moldave, 1969). Finally, in a study where the EF-Tu ternary complex was covalently cross-linked to the 70S ribosome in the presence of GMPPCP to prevent GTP hydrolysis, almost half of the covalently bound EF-Tu was found on the 30S subunit (San Jose et al., 1976). Unfortunately, the authors only analyzed the interactions with the proteins of the 50S subunit. Nevertheless, it appears that the EF-Tu ternary complex is binding to or interacts with a significant portion of the small subunit in both eukaryotes and prokaryotes.

The relative ease with which 50S subunits release EF-Tu from 30S complexes suggests that binding of the EF-Tu ternary complex does not significantly alter the region of the 30S subunit in contact with the 50S subunit. The results of the partial release experiments (shown in Figure 5) are quite clear. They detect no significant enrichment (or depletion) in the percentage of 30S subunits carrying bound EF-Tu when prebound 30S complexes are reacted with varying amounts of 50S subunits. In other words, a 30S subunit interacts equally well with a 50S subunit whether or not an EF-Tu ternary complex is bound to it. This suggests that the EF-Tu complex might not be present at the interface surface of the small subunit. It is interesting to note that it has been proposed (Lake, 1977), on the basis of the three-dimensional locations of ribosomal proteins involved in tRNA binding, that the recognition site, i.e., the ternary complex binding site, might be on the external or cytoplasmic side of the small subunit. Our data are consistent with that proposal.

Conclusions

We have demonstrated that elongation factor Tu can form a stable complex with the 30S ribosomal subunit in the presence of GTP, Phe-tRNA, and oligo(U). The ternary complex seems correct and nonartifactual in its dependence on oligo(U) and Phe-tRNA and in all of its subsequent reactions with the 50S ribosomal subunit. These reactions include the 50S-mediated hydrolysis of GTP, the 50S-mediated release of EF-Tu, and the coupling between these two reactions. Finally, we have presented evidence that proteins L7/L12 are not required for the 50S-mediated hydrolysis of GTP or the 50S-mediated release of EF-Tu, thereby demonstrating the usefulness of the 30S–EF-Tu complex for future studies of ribosome function.

These results, compared with those of other workers on the properties of EF-Tu binding to the 70S ribosome, lead us to conclude that (1) a significant part of the EF-Tu–aa-tRNA–GTP binding site is on the 30S ribosomal subunit, (2) the EF-Tu ternary complex is bound to the 30S subunit so that it does not inhibit binding of the 50S subunit, and (3) the EF-Tu ternary complex is bound to the 30S subunit in the functionally active site.

Added in Proof

Recently the binding site of EF-Tu has been localized by immune electron microscopy (J. A. Langer and J. A. Lake, unpublished results) using 30S–EF-Tu complexes. As the data in this paper suggest, the EF-Tu site is on the external surface of the 30S subunit.

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Processing of Preproteins by Liposomes Bearing Leader Peptidase†

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ABSTRACT: Procoat, the precursor form of M13 coat protein, assembles into sealed liposomes bearing only internally oriented leader peptidase and is processed to yield transmembrane coat protein [Ohno-Iwashita, Y., & Wickner, W. (1983) *J. Biol. Chem. 258*, 1895-1900]. The precursors of maltose-binding protein and of outer membrane protein A (OmpA) are also processed by these liposomes, showing that these preproteins can at least partially insert across a lipid bilayer. The ability to insert into a bilayer may be a general property of preproteins. The cleavage products, mature OmpA and maltose-binding protein, are not sequestered within the liposomes, suggesting that an additional factor(s) is (are) required for complete translocation. Liposomes were also prepared with leader peptidase in a more physiological, membrane-spanning orientation. These liposomes were also active in the cleavage of externally added procoat, pro-OmpA, and pre maltose-binding protein, though the mature OmpA and maltose-binding protein were still not sequestered within the liposomes. Pretreatment of these liposomes with trypsin cleaved near the amino terminus of the leader peptidase, inactivating the enzyme. The function of this amino-terminal domain, on the opposite side of the membrane from the catalytic domain, is unknown.

The assembly of proteins into (or across) membranes can be envisioned as occurring in three stages: (i) binding to the membrane, (ii) translocation across the apolar hydrocarbon core of the bilayer, and, often, (iii) covalent modification, as by the removal of an amino-terminal leader peptide. In bacteria, presecretory or membrane proteins are made either by free polysomes (Ito et al., 1979) or by membrane-bound polysomes (Randall & Hardy, 1977; Smith et al., 1979); proteins that may participate in binding certain nascent chains have been identified genetically (Michaelis & Beckwith, 1982) and biochemically (Horiuchi et al., 1983). Proteins such as M13 procoat (Date & Wickner, 1981a,b), pre-lam B protein (Josefsson & Randall, 1981), and pre-TEMβ-lactamase (Koshland & Botstein, 1982) are complete prior to translocation across the membrane. Others such as maltose-binding

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