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Isolation and stability of ternary complexes of elongation factor Tu, GTP and aminoacyl-tRNA

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ABSTRACT

Intact, native EF-Tu, isolated using previously described methods and fully active in binding GTP, was never found to be fully active in binding aminoacyl-tRNA as judged by high performance liquid chromatography (HPLC) gel filtration and zone-interference gel-electrophoresis. In the presence of kirromycin, however, all these EF-Tu.GTP molecules bind aminoacyl-tRNA, although with a drastically reduced affinity. For the first time, the purification of milligram quantities of ternary complexes of EF-Tu.GTP and aminoacyl-tRNA, free of deacylated tRNA and inactive EF-Tu, has become possible using HPLC gel filtration. We also describe an alternative new method for the isolation of the ternary complexes by means of fractional extraction in the presence of polyethylene glycol. In the latter procedure, the solubility characteristics of the ternary complexes are highly reminiscent to those of free tRNA. Concentrated samples of EF-Tu.GMPPNP.aminoacyl-tRNA complexes show a high stability.

INTRODUCTION

Although it was reported already 22 years ago that EF-Tu forms a complex with GTP and aminoacyl-tRNA (1) and promotes the attachment of the latter molecule to the ribosomal A-site (2), even today there seems to be no strict consensus on the actual stoichiometry of the complex (3). One of the reasons for this is the versatility of the protein: it has many reaction partners and it can adopt a large number of conformations (4). It has been shown that interactions with some of its partners can selectively be blocked by chemical modifications or by antibiotics (5,6). It was even reported that a fraction of an intact, native EF-Tu.GTP preparation was inactive in binding aminoacyl-tRNA (aa-tRNA), although it was fully able to bind the nucleotide (7). For many types of experiments such heterogeneity is of course detrimental and several techniques for the isolation of the ternary complex have therefore been described. One way to purify the complex

was reported in (8): after passing a mixture of EF-Tu.GTP and aa-tRNA through several layers of nitrocellulose membrane, the inactive fraction of EF-Tu remains bound to the membrane. However, free tRNA cannot be separated from this complex. Ternary complexes have also been purified on standard gel filtration columns (for example see (10)), which clearly separate the complex from monomeric tRNA. Unfortunately, the complexes proved to have about the same elution volume as free EF-Tu.GTP. In this paper we describe several other techniques to purify and analyse the ternary complex.

Both the instability of the nucleotide cofactor GTP and of the ester bond between the amino acid and the tRNA may reduce the stability of the complex of EF-Tu.GTP and aa-tRNA. For some types of experiments it is of vital importance that the complexes of EF-Tu.GTP and tRNA be as stable as possible. By making use of the non-hydrolysable analogue GMPPNP, one can circumvent the problems caused by hydrolysis of GTP. The aminoacyl ester bond in Val-tRNA^{Val} is one of the stablest of its kind (11). The aminoacyl ester bond of an aa-tRNA is stabilized in the complex with EF-Tu.GTP (9,12). By choosing a pH as low as possible, one can further increase its stability. In this paper we present observations concerning the durability of maximally stabilized complexes.

In attempts to crystallize the ternary complex of EF-Tu.GTP.aa-tRNA, we developed several techniques to purify and analyse it. We think that these techniques are of major importance to those who are engaged in functional studies on the interactions between EF-Tu and tRNA, and with their common target: the ribosome. Especially the paragraphs dealing with the solubility characteristics of the ternary complex might be interesting for investigators of protein-nucleic acid interactions in general.

MATERIALS AND METHODS

Guanine nucleotides, phosphoenolpyruvate, pyruvate kinase and bulk tRNA were purchased from Boehringer Mannheim. Agarose was purchased from Bethesda Research Laboratories, Neu Isenburg, FRG. [¹⁴C] Amino acids (50 mCi/mmol) used to

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check the yield of aminoacylation of tRNAs, as well as [^3H] GTP and GDP (both at 10 Ci/mmol) were from Amersham, England. Kirromycin was a gift from Gist-brocades, Delft, the Netherlands. Aquacide II was purchased from Hoechst. All other chemicals were from Merck, Darmstadt, FRG. PEG 6000 was gas chromatography grade.

EF-Tu.GDP from *E. coli* was prepared according to (13). All preparations of EF-Tu were homogeneous and intact as judged by SDS polyacrylamide gel electrophoresis and all were active for at least 90% as judged by the GDP exchange assay via nitrocellulose membrane filtration. The aminoacylation of bulk tRNA and complex formation with EF-Tu were according to (14), with the exception that the aminoacylated tRNA was not dialysed against reaction buffer, but was dissolved in 2 mM potassium acetate pH 5. Aminoacylated tRNA was stored at -70°C . Zone-interference gel electrophoresis was performed as in (15).

For the separation of ternary complexes by means of HPLC we used TSK-125 or TSK-250 silica-based gel filtration columns from either Biorad or LKB. Elution buffers containing 10 to 50 mM of either HEPES, PIPES, MOPS or sodium cacodylate pH 7.0, 150 mM KCl, 10 mM MgCl_2 and 1 mM NaN_3 all give a good separation. Lowering the concentration of monovalent ions causes broadening of the peaks without a drastic change in retention time.

RESULTS

Analysis of complexes of EF-Tu and aa-tRNA by native gel electrophoresis

Zone-interference gel electrophoresis, the method we used to examine weak complexes of tRNA and EF-Tu.kirromycin.GTP/GDP (15), can also be used to examine complex formation in the absence of kirromycin. Since the complex is quite stable in the presence of GTP (recent papers report dissociation constants in the sub-nanomolar range (7,16,17)), it is not surprising that a discrete band shift is observed: during the electrophoresis the complex hardly dissociates. Figure 1A shows that not all the EF-Tu in the

preparation was active in binding aa-tRNA and that this portion of inactive protein is not reactivated by a prolonged incubation in the presence of aminoacyl-tRNA and GTP. However, the EF-Tu preparation used for this experiment was fully active in binding the nucleotide as judged by nitrocellulose membrane filtration. Furthermore, EF-Tu purified by affinity chromatography on a GDP-Sepharose column (18) gave similar results. The best preparation of EF-Tu with respect to aa-tRNA binding we have examined up till now, was 80% active in forming a complex with aa-tRNA, as determined by zone-interference gel electrophoresis. It is striking that addition of kirromycin reactivates EF-Tu: the protein now displays a uniform affinity for aminoacyl-tRNA (see figure 1B and (15,19)).

The ternary complex seems to dissociate slowly upon prolonged incubation in 10% (v/v) DMSO as used for the samples analysed by zone-interference gel electrophoresis. Since HPLC gel filtration in the absence of DMSO confirms that preparations of EF-Tu can be only partially active in binding tRNA, it is unlikely that the results obtained with zone-interference gel electrophoresis were caused by an artifact. In the presence of kirromycin,

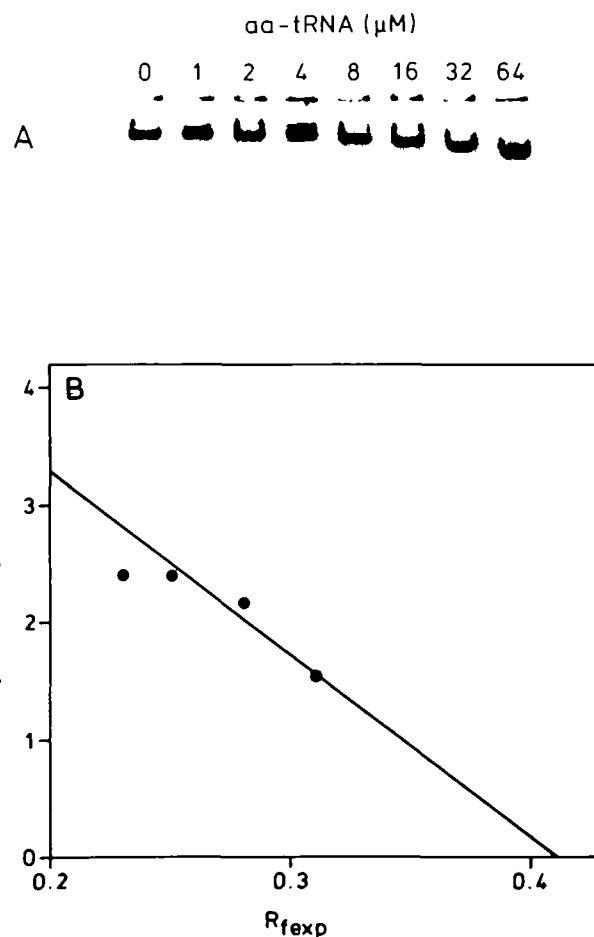


Figure 2. (A) Zone-interference gel electrophoresis of EF-Tu.GDP.aa-tRNA. The aminoacylated bulk tRNA was passed over a G-50 Sepharose column. The concentrations of aa-tRNA in the zones are indicated. For further details, see the legend of figure 1. The gel was stained with Coomassie Brilliant Blue. (B) Determination of the dissociation constant of EF-Tu.GDP.aa-tRNA as described in (5). Only the lanes with the four highest tRNA concentrations provide data for a more or less reliable calculation. The R_f values of the bands are calculated as their migration distance relative to that of bromo-phenol blue.

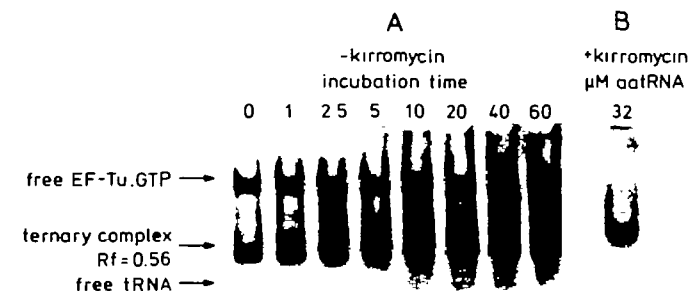


Figure 1. (A) Zone-interference gel electrophoresis of 5 pmols of EF-Tu.GTP.aa-tRNA complexes (10 μl) in 20 mM Tris.acetate pH 7.6, 3.5 mM MgCl_2 on a 2% agarose gel at 9°C . Besides buffer, the zone contained 10 μM of aminoacylated bulk tRNA, 10 μM of GTP, 10 μM PEP and 10 $\mu\text{g/ml}$ PK. Prior to electrophoresis 5 μM of EF-Tu.GDP was preincubated at 37°C with 500 μM GTP, 500 μM PEP, 100 $\mu\text{g/ml}$ PK and 15 μM aa-tRNA in buffer. The different lanes correspond to different incubation times. Immediately prior to electrophoresis 10% of DMSO was added to the samples. The gel was stained with Coomassie Brilliant Blue. (B) Zone-interference gel electrophoresis of EF-Tu.kirromycin.GTP.aa-tRNA. The procedure was the same as in panel (A), with the exception that the zone now contained 32 μM of aa-tRNA.

complex formation is not affected by the presence of DMSO (not shown).

We have also examined complexes of EF-Tu.GDP.aa-tRNA with zone-interference gel electrophoresis. In a preliminary report (19), we mentioned the possibility of stable complexes which only seemed to form at high concentrations of aa-tRNA. It is not excluded, however, that these complexes in fact contained GTP. The observed phenomenon was not caused by a GTP contamination in the GDP stock, since raising the GDP concentration decreased the amount of complex. However, after passing the aminoacyl-tRNA preparation over a G-50 column, the complexes disappeared (see figure 2A). Presumably the tRNA preparation contained a small amount of GTP, which must have been a contaminant of the ATP used to aminoacylate the tRNA and which was not removed by triple ethanol precipitation.

For the complex of EF-Tu.GDP.aa-tRNA, a dissociation constant of about $60 \mu\text{M}$ at 9°C can be calculated (see figure 2B). The graph extrapolates to a maximal relative migration

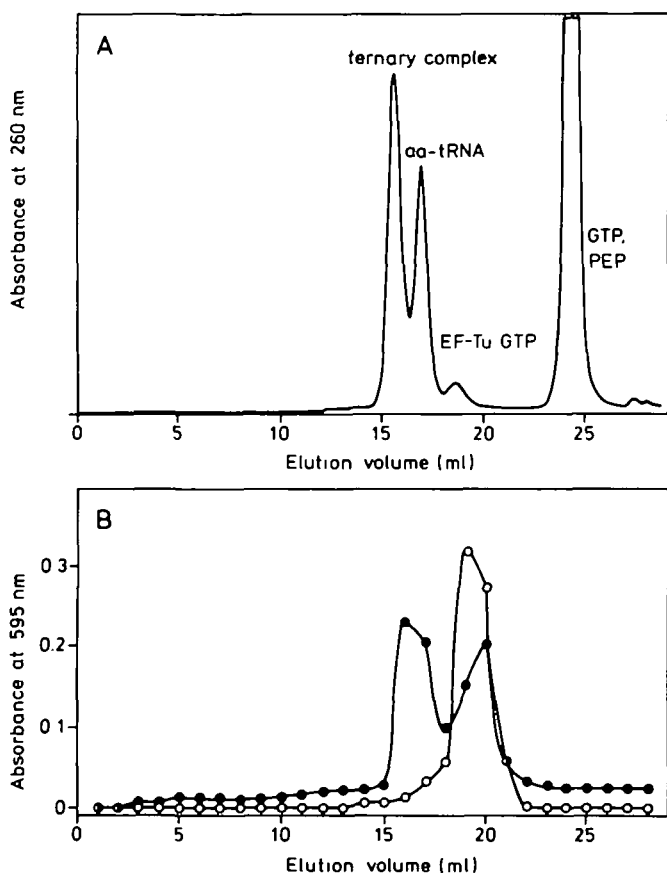


Figure 3. (A) Separation of EF-Tu.GTP.aa-tRNA complexes on an HPLC TSK-125 gel filtration column (7.5mm $\times$ 60 cm) at room temperature. A mixture of 2.7 nmol of partially aminoacylated bulk tRNA, 4.2 nmol of EF-Tu.GDP, 400 nmol PEP, 400 nmol GTP and $10 \mu\text{g}$ PK in $50 \mu\text{l}$ buffer containing 20 mM HEPES pH 7.0, 10 mM MgCl_2 , 150 mM KCl, was preincubated at 37°C for 15 minutes. The sample was eluted in the same buffer at a flow rate of 1 ml/min. The peaks of EF-Tu, aa-tRNA and GTP were identified in separate runs. (B) Mixtures of 4.2 nmol EF-Tu.GTP ($180 \mu\text{g}$), 400 nmol PEP, 400 nmol GTP and $10 \mu\text{g}$ PK in the same elution buffer were added to 14 nmol deacylated tRNA (open circles) or 14 nmol aa-tRNA (closed circles) to a total volume of $50 \mu\text{l}$. After incubation at 37°C for 15 minutes, the mixtures were applied to the TSK-125 column. The EF-Tu distribution over the fractions (0.5 ml) was determined with a Bradford protein assay.

distance different from that of EF-Tu.GTP.aa-tRNA, which could indicate a different net charge or a different conformation of the complex containing GDP. A difference in charge might at least be partially explained by the absence of the γ -phosphate in the complex with GDP. When we substituted aa-tRNA by HO-tRNA or when we substituted EF-Tu.GDP by ovalbumin, we observed similar electrophoretic patterns (not shown). The actual value we report for the dissociation constant of a complex of EF-Tu.GDP and bulk aa-tRNA could therefore be argued. Nevertheless, it compares quite well with the one of the complex of EF-Tu.GDP.Phe-tRNA^{Phe}-Fl⁸ reported in (20), which was about $30 \mu\text{M}$ at 6°C . It is a bit higher than the dissociation constant of a similar complex without the fluorescence label, which was reported to be $14 \mu\text{M}$ at 25°C , as measured with a hydrolysis protection assay (21).

Purification of EF-Tu.GTP.aa-tRNA complexes by HPLC

We tried to purify the ternary complex from its inactive components in several ways. Preparative native gel electrophoresis in agarose resulted in a good separation, but it proved too cumbersome to elute the complexes from the gel. By means of stepwise elution of a DEAE-Sephacel column, we were not able to separate the ternary complex from free tRNA. We used a buffer containing 20 mM HEPES pH 7.05, 10 mM MgCl_2 , 1 mM NaN_3 , $10 \mu\text{M}$ PMSF. Both the ternary complex and tRNA elute at 650 mM KCl in this buffer. However, we did get success with Biosil TSK-125 and TSK-250 HPLC gel filtration columns. Although these columns separate macromolecules mainly according to their size, we found that probably also the charge of the macromolecule significantly influences the elution volume. As is shown in figure 3A, aa-tRNA elutes prior to EF-Tu.GTP, although it is smaller. We made use of this observation to separate the ternary complex from free EF-Tu.GTP and free tRNA: the latter two components have a larger retention time on the column than the ternary complex. Ternary complexes isolated in this manner remain intact when reinjected

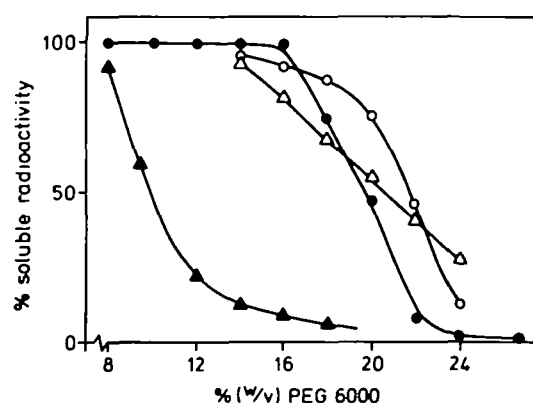


Figure 4. Solubility of ternary complexes at 0°C as a function of the concentration of PEG 6000. Samples containing $165 \mu\text{M}$ EF-Tu. ^3H GTP (Δ) and $130 \mu\text{M}$ [^{14}C]Val-tRNA^{Val} ($\circ$) in 10 mM HEPES pH 7.0, $20 \mu\text{M}$ GTP, $200 \mu\text{M}$ PEP were brought to various concentrations of PEG 6000. The precipitates were spun down and the radioactivities of the supernatant and the pellet were determined separately. The tritium counts of the GTP are assumed to be associated with EF-Tu. Closed symbols show the behaviour of the single components, as displayed in control experiments in which either the EF-Tu or tRNA was omitted. Open symbols refer to the behaviour of EF-Tu and tRNA if both are present at the same time.

in sufficiently high concentrations on the gel filtration column, as judged by the elution profile. The separation of the complex from the constituting monomers is influenced by the absolute amount of complex applied. When 1 nmole of ternary complex in 50 μ l is injected, about half of it stays intact, while the rest dissociates. When a larger amount is applied, a higher proportion stays in complex. The dissociated aa-tRNA shows up as a clear separate peak, having about the same width as the one of the ternary complex. We found that the addition of 10 μ M GTP to the elution buffer marginally improves the separation of the ternary complex from tRNA. Low concentrations of GDP in the sample (even down to one tenth of the GTP-concentration) severely decrease the amount of ternary complex as judged by the HPLC profiles, a fact hard to explain in view of the high affinity of EF-Tu.GTP for aa-tRNA.

In agreement with zone-interference gel electrophoresis, none of the EF-Tu preparations we used was 100% active in binding aa-tRNA as judged by HPLC gel filtration. An example is shown in figure 3B.

Purification of EF-Tu.GTP.aa-tRNA complexes by fractional extraction

In attempts to crystallize ternary complexes of EF-Tu.GTP.aa-tRNA we made use of the relatively stable Val-tRNA^{Val} from *E. coli* (11). Precipitation studies with increasing concentrations of PEG-6000 indicate that the ternary complex of EF-Tu.GTP and Val-tRNA^{Val} has similar solubility characteristics as Val-tRNA^{Val} alone (see figure 4). The solubility of EF-Tu.GDP was unaffected by the presence of the charged tRNA. Furthermore, only the charged tRNA was capable of increasing the solubility of EF-Tu.GTP. This can be readily understood from the marked increase in charge of the ternary complex as compared to that of EF-Tu.GTP (cf. figure 1). Another indication for the substantial net negative charge of the ternary complex is that we were not able to separate it from free tRNA on a DEAE-Sephacrose column (see previous paragraph).

The observations described above prompted the separation of the ternary complex from EF-Tu.GTP inactive in binding aa-tRNA by first precipitating EF-Tu.GTP with PEG 6000 or ammonium sulphate and subsequent addition of a smaller molar amount of charged tRNA. If the PEG or ammonium sulphate concentration is high enough to precipitate free EF-Tu, but too low to precipitate the ternary complex, an amount of protein equimolar to the amount of charged tRNA will dissolve. In a typical experiment we precipitated 55.2 nmol EF-Tu.GTP in a buffer containing 14% PEG, 10 mM MOPS pH 7.0, 30 mM KCl, 10 mM MgCl₂, 0.5 mM DTT, 1 mM NaN₃, 2.7 mM GMPPNP, 5.6 mM PEP, 10 μ g pyruvate kinase (total volume 185 μ l). We added 55 μ l containing 22 nmol Val-tRNA^{Val} in the same buffer. A portion of the EF-Tu now became soluble. HPLC analysis of the soluble fraction confirmed that all the aminoacylated tRNA (90% of the total amount of tRNA) was in complex with EF-Tu. A Bradford assay indicated that the soluble fraction contained 6.6 mg/ml of protein. A separate measurement of the optical density at 260 nm of a 100 times diluted sample of the soluble fraction, measured against a 1:100 dilution of the (GMPPNP containing) buffer used, indicated that this fraction contained 106 A₂₆₀ units of tRNA^{Val} per ml. Therefore, the soluble fraction contained 1.1 mole of tRNA per mole of EF-Tu (1 A₂₆₀ unit of pure tRNA^{Val} corresponds to 1.6 nmol). Although the actual concentrations of monovalent and divalent ions as well as the pH and nature of the buffer are important determinants of the solubility of the complex, the

procedure works over a range of conditions and for various precipitants. Care must be taken with the use of polyelectrolytes like spermine, since in the case of Val-tRNA^{Val}, 2 mM of spermine seems to dissociate the ternary complex. Mistakes can easily be made since spermine was observed to decrease the elution volume of tRNA^{Val} on the TSK-125 gel filtration column, thus making the tRNA appear to be engaged in ternary complex formation. A similar behaviour of other tRNA species cannot be excluded.

We found some factors determining the content of partially active EF-Tu molecules in our preparations. Concentration of EF-Tu.GDP containing fractions by precipitation with ammonium sulphate at 50% saturation after the DEAE-column and the ACA 44 gel filtration column during the isolation gives rise to more active preparations with respect to aa-tRNA binding than precipitation at 70% saturation. An alternative method of protein concentration by means of dialysis bags placed in Aquacide II, also yields a preparation less active in tRNA binding. At the end, either method can yield electrophoretically pure preparations, which are for 90 to 100% active in binding GDP. This suggests that the fraction of EF-Tu inactive in binding tRNA is more soluble in ammonium sulphate than active EF-Tu.

Stability of EF-Tu.GMPPNP.aa-tRNA complexes

An easy test for the intactness of ternary complexes is suggested by the large difference between the solubility of the ternary complex and that of EF-Tu.GTP. A functional interaction between EF-Tu.GTP and aa-tRNA may be assumed when a mixture of them remains clear in a buffer containing a precipitant at a concentration which would normally precipitate a comparable concentration of free EF-Tu.GTP. In this paragraph two individual examples are given.

During crystallization trials, we observed that a mixture of about 10 mg/ml of EF-Tu.GMPPNP.Val-tRNA^{Val} in 10 mM MOPS pH 7.0, 10 mM MgCl₂, 30 mM KCl, 1 mM NaN₃, 500 μ M DTT, 1.2 mM GMPPNP, 14% PEG 6000 (w/v) remained clear with a completely intact ternary complex as judged by HPLC analysis after 6 days storage at 4°C. Furthermore, upon prolonged storage of a different Phe-tRNA-containing ternary complex under quite different conditions (about 10 mg/ml of EF-Tu.GTP.Phe-tRNA^{Phe} in 12.5 mM sodium cacodylate pH 7.0, 5 mM MgCl₂, 5 mM β -mercaptoethanol, 3 mM GTP, 3 mM PEP, 30 μ g/ml pyruvate kinase and 33% saturated ammonium sulphate) the mixture even remained clear for 7 months at 4°C. A control experiment containing exactly the same components, with the exception of Phe-tRNA^{Phe} being replaced by deacylated tRNA^{Phe}, never produced a clear mixture. For unknown reasons, however, we could not detect ternary complexes in the ammonium sulfate containing mixture which had remained clear. From the literature it is known that in the absence of PEG or ammonium sulphate and at low concentrations of EF-Tu and aa-tRNA the ternary complex has a much shorter half-life (12).

DISCUSSION AND CONCLUSIONS

With several techniques we were able to purify and analyse complexes of EF-Tu.GTP and aa-tRNA. When the concentrations of the components could be measured, we found a 1:1 stoichiometry of the complex. The techniques described in this paper yielded a few surprises, the main one being that preparations of EF-Tu can be completely active in binding the nucleotide, but only partially in binding aa-tRNA. This could be one of the causes of the great diversity in dissociation constants

reported for the ternary complex by various groups (see for example (12) and (16)) and the deviating stoichiometry reported in (3). We do not exclude that the GTPase activity of EF-Tu molecules inactive in tRNA-binding, but still capable of GTP-binding, can be stimulated by ribosomes. Such an artifact could be an additional explanation for the observations reported in (3) which indicate a non-stoichiometric ribosome induced GTP hydrolysis of EF-Tu with respect to peptide bond formation.

It is surprising that ternary complexes seem to be dissociated by small quantities of GDP as judged by HPLC gel filtration. A twofold excess of GDP over GTP should result in a 1:1 ratio of EF-Tu.GTP.aa-tRNA and EF-Tu.GDP if the total concentrations of both EF-Tu and aa-tRNA are in the micromolar range. This can be inferred from the dissociation constant of the EF-Tu.GTP.aa-tRNA complex which is about 10^{-9} M (7,16,17) and from the fact that EF-Tu has about 500-fold higher affinity for GDP than for GTP (22), in the following way:

$$K_1 = \frac{[\text{EF-Tu}][\text{GDP}]}{[\text{EF-Tu.GDP}]} \quad (1)$$

$$K_2 = \frac{[\text{EF-Tu}][\text{GTP}]}{[\text{EF-Tu.GDP}]} \quad (2)$$

$$K_3 = \frac{[\text{EF-Tu.GTP}][\text{aa-tRNA}]}{[\text{EF-Tu.GTP.aa-tRNA}]} = 10^{-9}\text{M} \quad (3)$$

$$\frac{K_2}{K_1} = 500 \quad (4)$$

From (1), (2), (3), and (4) follows

$$\begin{aligned} \frac{[\text{aa-tRNA}][\text{EF-Tu.GDP}]}{[\text{EF-Tu.GTP.aa-tRNA}]} &= \frac{K_2 K_3}{K_1} \cdot \frac{[\text{GDP}]}{[\text{GTP}]} \\ &= 0.5 \frac{[\text{GDP}]}{[\text{GTP}]} \cdot 10^{-6}\text{M} \quad (5) \end{aligned}$$

Now suppose that 2 μM of EF-Tu and 2 μM of tRNA are present, and that half of the EF-Tu is in complex with the tRNA. Equation (5) indicates that in this case equilibrium conditions are met with $[\text{GDP}]/[\text{GTP}] = 2$.

If the observed behaviour is not caused by artifacts, it could be that in the conditions used for the purification of the complex on TSK gel filtration columns, the ratios between the equilibrium constants of EF-Tu.GTP, EF-Tu.GDP and EF-Tu.GTP.aa-tRNA are altered.

The similar solubility characteristics of aa-tRNA and the ternary complex are explained by the large net negative charge of both. The similarity in charge is reflected by the high electrophoretic mobility of the complex, which, despite the considerably larger size of the complex, is more comparable to that of aa-tRNA than to that of EF-Tu.GTP (cf. figure 2A). Also the fact that we were not able to separate ternary complexes from free tRNA on a DEAE-Sepharose column indicates the high similarity in charge of the ternary complex and free tRNA. We observed that spermine decreases the solubility of the ternary complex to a similar extent as that of the free tRNA, although we cannot completely rule out that spermine precipitates the free components rather than the ternary complex. The fact that millimolar concentrations of spermine eventually dissociate the ternary complex of EF-Tu.GTP.Val-tRNA^{Val} suggests that contacts between the phosphates of the tRNA and positively charged residues of the protein (like Lys or Arg), are stabilizing the

complex, although spermine might also induce a conformation of the tRNA not recognized by EF-Tu.

The experiments reported in this paper indicate a high stability of the ternary complex. We suspect that the increase in stability of the aminoacyl ester bond and hence of the half-life of the ternary complex was mainly caused by the increase in concentration of the macromolecules. Furthermore, it was shown that also precipitants have a stabilizing effect (23).

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