The influence of tRNA located at the P-site on the turnover of EF-Tu·GTP on ribosomes

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The influence of tRNA located at the P-site on the turnover of EF-Tu•GTP on ribosomes

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Summary — The turnover of EF-Tu•GTP on poly-U programmed ribosomes was measured both in the presence and in the absence of N-acetylated Phe-tRNA^{Phe} at the P-site. The reaction was uncoupled from protein synthesis by omitting Phe-tRNA^{Phe} at the A-site. In this reaction, the ribosome can be considered as an enzyme catalysing the transition of EF-Tu•GTP to EF-Tu•GDP. A constant EF-Tu•GTP concentration is maintained by regenerating GDP to GTP at the expense of phosphoenolpyruvate by pyruvate kinase. The rate constants are determined using a procedure which corrects for the reduction in specific activity of GTP due to regeneration of the nucleotide. Ribosomes with an occupied P-site are more efficient in stimulating the GTPase of EF-Tu•GTP than ribosomes with an empty P-site. The data suggest that this is mainly caused by an increased affinity of EF-Tu•GTP for ribosomes with a filled P-site rather than by an enhanced reactivity of the GTPase centre.

elongation factor Tu / GTPase activity / ribosome / P-site / ribosome–EF–Tu interaction

Introduction

The elongation factor Tu (EF-Tu) promotes the attachment of aminoacylated tRNA (aa-tRNA) at the ribosomal A-site. The increased affinity of tRNA with an anticodon matching the codon of the messenger causes the ternary complex to pause long enough on the ribosome to allow EF-Tu hydrolyse its nucleotide co-factor GTP to GDP and phosphate. Only after hydrolysis does EF-Tu•GDP leave the ribosome–tRNA complex, thereby permitting peptide bond formation. It has been shown that the GTP-hydrolysis is a crucial step in peptide elongation, being the driving force of proofreading of the tRNA. If the cofactor is replaced by the non-hydrolysable analogue GMPPNP no peptide bond formation occurs, although the affinity of the ternary complex for the ribosomal A-site is not affected. This indicates that peptide bond formation can only occur after EF-Tu has left the ribosome. If GTP is replaced by the slowly hydrolysing GTPγS, the slowed down translation process takes place with an increased fidelity. This indicates that the duration of the pause of the ternary complex on the ribosome is directly related to the fidelity of translation (for a review, see [1, 2]).

It is known that the affinity of the ternary complex for ribosomes is increased by the presence of uncharged tRNA at the P-site [3, 4]. Such an increase in affinity could be due to contacts between the tRNAs at the A-site and the P-site in view of reports on dimerisation of tRNAs in the presence of oligonucleotides complementary to their anticodon [5]. Contacts between EF-Tu and tRNA located at the ribosomal P-site could be another cause of the increased affinity. Such a contact was shown in our laboratory by means of cross-linking [3]. In the absence of tRNA, the GTPase activity of EF-Tu is known to be markedly increased by ribosomes [6]. The presence of tRNA at the ribosomal P-site causes further stimulation of the GTPase of EF-Tu when engaged in ternary complex [7]. The data in [7] also indicate that the presence of tRNA at the ribosomal P-site does not have an effect on the ribosome stimulated GTPase of EF-Tu. However, the data presented in our report do suggest such an effect. We address the question how this stimulation takes place: does peptidyl-tRNA increase the affinity of EF-Tu•GTP for the ribosomal complex, or does peptidyl-tRNA assist the ribosome in allosterically changing EF-Tu and thereby inducing the factor to hydrolyse GTP faster (as was postulated in [8])?

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Materials and methods

All the materials and the preparation of N-acetyl-Phe-tRNA^{Phe} (NacPhe-tRNA^{Phe}) were as described in [8]. EF-Tu and EF-Ts were isolated according to [9]. Both were homogeneous and intact as judged by SDS gel electrophoresis and EF-Tu was fully active in GDP binding as determined by nitrocellulose membrane filtration. Tight couples of ribosomal subunits were isolated from NH_4Cl-washed ribosomes by centrifugation in a sucrose gradient (10–30%) in 10 mM Tris–HCl pH 7.6, 5 mM $MgCl_2$, 30 mM NH_4Cl, 6 mM β-mercaptoethanol. They proved to be 80% active in poly-U programmed binding of Nac[^{14}C]Phe-tRNA^{Phe}. We could not detect EF-G contamination in any of the preparations used since inclusion of fusidic acid did not influence the ribosome stimulated GTPase.

Mixtures containing ribosomes (1.4 μM), poly-U (1.8 mg/ml) and in some cases NacPhe-tRNA^{Phe} (3.5 μM) in 14.1 mM Tris–HCl pH 7.6, 14.1 mM $MgCl_2$, 120 mM NH_4Cl and 5.3 mM β-mercaptoethanol, were incubated at 42°C for 15 min and subsequently at 37°C for 5 min immediately before use. A mixture containing 130 μM EF-Tu, 3.2 μM EF-Ts, 21 μM pyruvate kinase, 1.8 mM GTP (or 5.5 mM GTP if ribosomes filled at the P-site were added) and 15 mM phosphoenolpyruvate (PEP) in buffer A (20 mM Tris–HCl pH 7.6, 4 mM $MgCl_2$, 60 mM KCl and 4 mM β-mercaptoethanol) was diluted to various concentrations using buffer A and to 4 μl of each of the resulting mixtures 1 μl of [γ-^{32}P]-GTP (185 TBq/mmol, 20–40 MBq/ml) was added. These mixtures were incubated at 37°C for 5 min and to each of these mixtures 5 μl of the ribosomes equilibrated at 37°C was added. This means that the GTPase reaction took place in 15 mM Tris–HCl pH 7.6, 8.6 mM $MgCl_2$, 60 mM NH_4Cl, 25 mM KCl, 4.3 mM β-mercaptoethanol. After 5 min at 37°C 3-μl samples were spotted on a poly-ethyleneimine thin layer plate that was eluted with 0.5 M formic acid and 0.5 M LiCl. After autoradiography, the spots of $^{32}P_i$ and [γ-^{32}P]-GTP were cut out and subjected to liquid scintillation counting.

Results

The turnover of EF-Tu•GTP on poly-U programmed ribosomes was measured with the help of the system depicted in figure 1. The ribosome can here be considered as an enzyme which catalyses the transition of EF-Tu from a non-hydrolysing conformation to one which hydrolyses GTP quickly. This reaction is coupled to GDP/GTP exchange on EF-Tu by EF-Ts and to regeneration of GDP by PK and PEP. The following equation* relates the amount of [γ-^{32}P]-GTP remaining after a certain incubation time to the total amount of hydrolysed GTP. The equation takes into account that the specific radioactivity of GTP decreases during the reaction because an excess of non-labelled PEP is used to regenerate hydrolysed GTP.

$$[P_i] = [\mathrm{GTP}] \ln \frac{\mathrm{cpm}_{tot}}{\mathrm{cpm}_t}$$

$[P_i]$: amount of phosphate produced during time t
[GTP]: total amount of GTP present either free or complexed (please note that this is amount

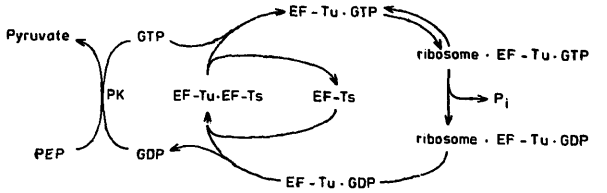

Fig 1. System with which the turnover of EF-Tu•GTP on ribosomes can be measured. After GTP-hydrolysis on the ribosome, EF-Tu•GDP and phosphate are released. EF-Tu•GDP is regenerated to EF-Tu•GTP by EF-Ts, GDP is phosphorylated by pyruvate kinase at the expense of phosphoenolpyruvate to produce GTP. The reaction is started with an excess of GTPγ[^{32}P] over EF-Tu. After a certain period, GTP-hydrolysis is determined by measuring the ratio between free [^{32}P]-phosphate and [γ-^{32}P]-GTP. Since phosphoenolpyruvate is not labelled, the specific activity of GTP decreases during the incubation. Correction for this decrease is pointed out in the text.

*The equation given in the text can be derived from equations (1), (2), and (3) (radioactive GTP is marked with an asterisk; other concentrations are given irrespective of their ^{32}P contents; concentrations which vary in time are in italics):

$$\frac{[\mathit{EF\text{-}Tu{\cdot}GTP{*}{\cdot}ribosome}]}{[\mathrm{EF\text{-}Tu{\cdot}GTP{\cdot}ribosome}]} = \frac{[\mathit{GTP{*}}]}{[\mathrm{GTP}]} \quad (1)$$

(Since EF-Tu cannot discriminate between labelled and non-labelled GTP)

$$\frac{\delta[P]}{\delta t} = k_{cat}\,[\mathrm{EF\text{-}Tu{\cdot}GTP{\cdot}ribosome}] \quad (2)$$

(By definition)

$$\frac{\delta[\mathit{GTP{*}}]}{\delta t} = -k_{cat}\,[\mathit{EF\text{-}Tu{\cdot}GTP{*}{\cdot}ribosome}] \quad (3)$$

(Since labelled and non-labelled GTP are hydrolyzed at the same rate), [*EF-Tu•GTP*•ribosome*] in equation (3) can be substituted using equation (1):

$$\frac{\delta[\mathit{GTP{*}}]}{\delta t} = -k_{cat}\,[\mathrm{EF\text{-}Tu{\cdot}GTP{\cdot}ribosome}]\,\frac{[\mathit{GTP{*}}]}{[\mathrm{GTP}]} \quad (4)$$

The term (k_{cat} [EF-Tu•GTP•ribosome]) in equation (4) can be substituted using equation (2). This yields after rearranging:

$$\frac{[\mathrm{GTP}]}{[\mathit{GTP{*}}]}\,\frac{\delta[\mathit{GTP{*}}]}{\delta t} = -\frac{\delta[P]}{\delta t} \quad (5)$$

After multiplying both sides of (5) with (δt) and integrating (please note that [GTP] is constant, while [*GTP**] and [*P*] are mutually dependent), the equation given in the text results after substitution of the ratio ([GTP*$_o$]/[GTP*$_t$]) with (cpm_{tot}/cpm_t).

constant during the reaction because hydrolysed GTP is regenerated).

cpm_{tot} : total amount of radioactivity (corrected for background)

cpm_t : remaining radioactivity of [γ-^{32}P]-GTP at time t (corrected for background).

In separate experiments we made sure that in fact the ribosome-induced GTP hydrolysis was the rate limiting step by varying the concentrations of PK and of EF-Ts. We determined the Michaelis-Menten constant (K_m) and the rate constant (k_{cat}) of the turnover reaction of EF-Tu•GTP on the ribosome both in the presence and in the absence of NacPhe-tRNA^{Phe} at the P-site. Please note that the K_m of this reaction is determined by the affinity of EF-Tu•GTP for ribosome, rather than by the affinity of GTP for EF-Tu. Therefore, the values we report should not be compared to those reported in [10], wich is a measure for the affinity of EF-Tu for GTP in the presence of ribosomes. The k_{cat} of the reaction defines the maximal turnover rate of EF-Tu•GTP on the ribosome. The constants were measured by titrating ribosomes with increasing amounts of EF-Tu•GTP in the presence of an excess of GTP at 37°C. After a certain time, the amount of GTP hydrolysis was measured.

A double reciprocal plot of the titration data (fig 2) indicates a K_m of $\approx$ 200 µM and a k_{cat} of $\approx$ 7 s^{-1} in the case of ribosomes with a empty P-site. The values become approximately 40 µM and 9 s^{-1}, respectively, when the P-site is occupied by NacPhe-tRNA^{Phe}.

Discussion and conclusions

In this paper we describe the ribosome-stimulated GTPase reaction of EF-Tu•GTP uncoupled from translation by using ribosomes with an empty A-site. Because hydrolysed GTP is regenerated, multiple turnover of individual EF-Tu molecules is allowed. The ensuing reduction in specific activity of the nucleotide can easily be corrected for, as is shown in the text. The data suggest that the presence of peptidyl-tRNA at the ribosomal P-site stimulates the uncoupled GTPase reaction of EF-Tu•GTP at high concentrations of EF-Tu. This is not in agreement with data presented in [7], which did not reveal an effect of the presence of NacPhe-tRNA^{Phe} at the P-site on the ribosome stimulated GTPase, unless the A-site was occupied by Phe-tRNA^{Phe} and the GTPase was coupled to peptide bond formation. The results in [7] were measured in the presence of an excess of ribosomes with partially filled P-sites over EF-Tu, while we used an excess of EF-Tu over ribosomes. Because in the experiments described in our paper furthermore a small excess of NacPhe-tRNA^{Phe} over ribosomes was used, we cannot completely exclude the possibility that the stimulation we observe was in fact caused by simultaneous occupation of the ribosomal P-and A-site by NacPhe-tRNA^{Phe} and a contamination of deacylated tRNA, respectively. On the other hand, P-site bound NacPhe-tRNA^{Phe} excludes additional NacPhe-tRNA^{Phe} binding at the A-site [11] and there is no coupling to peptide bond formation in the present case. If the stimulating effect is uniquely caused by the presence of NacPhe-tRNA^{Phe} at the P-site, this effect is mainly due to an increased affinity of EF-Tu•GTP for ribosomes with an occupied P-site and less to an increased rate constant. The values we report for K_m and k_{cat} of the turnover EF-Tu•GTP on ribosomes carrying NacPhe-tRNA^{Phe} at the P-site are significantly higher than those reported in [12], which were measured at 5°C and in different buffer (4.6 µM and 0.17 s^{-1} respectively). Advantages of our system are that accumulation of EF-Tu•GDP is prevented and that EF-Tu•GTP free from excess GTP does not have to be prepared by addition of GTP to lyophilized nucleotide-free EF-Tu, which could cause inactivation. It is interesting to note that the k_{cat} value we report is close to the rate constant for peptide bond formation which *in vivo* is 15–20 s^{-1} [13]. In the presence of Phe-tRNA^{Phe} at the A-site, the value of k_{cat} at 5°C was reported to be 20 s^{-1} [14].

In [3] it was shown that the presence of uncharged tRNA^{Phe} at the P-site hardly increases the association

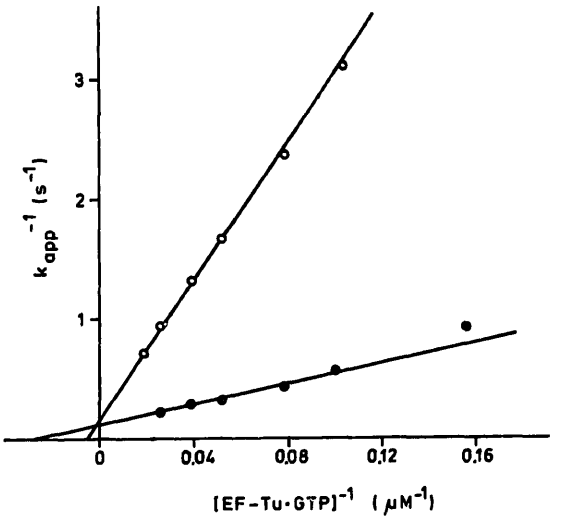

Fig 2. Determination of k_{cat} and K_m of the turnover of EF-Tu•GTP on poly-U programmed ribosomes. The reciprocal value of the measured turnover rate (k_{app}) of EF-Tu•GTP on ribosomes with a vacant P-site (open symbols), or with NacPhe-tRNA^{Phe} present at the P-site (closed symbols) is plotted *versus* the reciprocal concentration of EF-Tu•GTP. For experiment details, see *Materials and methods.*

rate of Phe-tRNA^{Phe} with poly-U programmed ribosomes unless EF-Tu•GTP is present. In the latter case, association is ≈ 10-fold faster. It was suggested that the increased association rate of ternary complexes is mainly due to contacts between EF-Tu and the tRNA at the P-site. Our present data support this hypothesis, since we observe a 5-fold decrease in K_m of the uncoupled turnover of EF-Tu•GTP on the ribosome, indicating that EF-Tu•GTP does not need to be complexed with aa-tRNA to display an increased affinity for ribosomes with a filled P-site. A cross-link between EF-Tu and periodate-oxidized tRNA^{Phe} located at the ribosomal P-site reported in [3] already indicated a contact between these molecules. A cross-link does not indicate the function of such a contact, however, and at the time it was also speculated that the interaction between the ternary complex and P-site bound tRNA was the trigger causing EF-Tu to hydrolyse GTP, pointing to an effect on the k_{cat}. Although we did not study the complete ternary complex, our present results rather indicate an effect on the K_m of the reaction.

The interaction of EF-Tu•GTP•aa-tRNA complexes with peptidyl-tRNA located at the ribosomal P-site could be one of the means by which ternary complexes discriminate between translating ribosomes which have not yet reinitiated and are scanning the messenger for an appropriate start [15].

In view of the relatively low affinity of EF-Tu•GTP for the ribosome, the uncoupled GTPase reaction will hardly take place *in vivo*. It is interesting to note that the equimolar amount of aa-tRNA and EF-Tu•GTP normally present in cells and the low dissociation constant of their ternary complex, effectively prevent the uncoupled and therefore non-productive GTP-hydrolysis of EF-Tu.

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