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Catalytic effects of elongation factor Ts on polypeptide synthesis

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The kinetic parameters which characterize the interaction between elongation factor Tu (EF-Tu) and elongation factor Ts (EF-Ts) have been determined in a poly(uridylic acid)-primed translation system. The EF-Ts catalyzed release of GDP from EF-Tu was measured independently in a nucleotide exchange assay. We conclude that the rate-limiting step for the EF-Tu cycle in protein synthesis in the absence of EF-Ts is the release of GDP. By adding EF-Ts the time of this step is reduced from 90 s to 30 ms. Half maximal rate is obtained at an EF-Ts concentration of 2.5 x 10^{-6} M.

Key words: EF-Ts/EF-Tu/kinetics/protein synthesis

Introduction

Protein synthesis in *Escherichia coli* takes place at an average rate of the order of 10–20 peptide bonds per second per ribosome at 20–37°C (Kennel and Riezman, 1977; Kjeldgaard and Gausing, 1974). One partial reaction in this process is carried out by elongation factor Tu (EF-Tu), which is bound to the ribosome as a ternary complex with GTP and aminoacyl-tRNA, and leaves the ribosome as a binary complex with GDP (Weissbach, 1980). The spontaneous dissociation of GDP from binary complex *in vitro* takes place at rates that are several orders of magnitude slower than the rate of peptide bond formation, but the addition of elongation factor Ts (EF-Ts) stimulates the regeneration of the ternary complex (Miller and Weissbach, 1970a). Studies performed in the absence of the other components of the translation system suggest that the dissociation rate of binary complex in the presence of EF-Ts might be as slow as 2 s^{-1} according to one group (Miller and Weissbach, 1977) and as fast as 1.7 x 10^3 s^{-1} according to a more recent study (Chau *et al.*, 1981).

We have taken advantage of a newly developed *in vitro* system that translates poly(uridylic acid) (poly(U)) at a rate comparable to that of the rate of peptide formation *in vivo* in order to measure the cycle time of EF-Tu in protein synthesis and in particular the influence of EF-Ts on this partial reaction. Our data show that EF-Ts stimulates the rate of generating EF-Tu·GTP by a factor of 2 x 10^3 and that the cycle time for the EF-Tu–EF-Ts interaction is only 30 ms. Comparison of these data with those obtained in a nucleotide exchange assay suggests that the release of GDP from the EF-Tu is indeed the reaction stimulated by EF-Ts in protein synthesis.

Results

Translation assay

It is possible to drive the elongation of polyphenylalanine (poly(Phe)) in a poly(U)-primed system at a rate of eight peptide bonds per second per ribosome if the ribosomes are initiated in a preincubation step (Wagner *et al.*, 1981). One of the requirements for this fast elongation rate is the presence of high concentrations of elongation factors which permits the kinetics of the process to be determined by the activity of ribosomes. To study the activity of one of the elongation factors it is similarly necessary to arrange the system so that all other components are present at relatively high concentrations, and the factor in question is at a concentration that limits the rate of elongation. Since the present study is concerned with the partial cycle of EF-Tu and the influence of EF-Ts upon this cycle, the construction of the appropriate incubation mixtures is somewhat delicate; thus, we are obliged to balance the concentrations of these two factors so that EF-Ts is kept at a concentration so low that it limits the overall rate of EF-Tu function. At the same time we require that the EF-Tu function limits the overall rate of poly(Phe) elongation.

The course of poly(Phe) elongation under such conditions is biphasic with an initial steep slope which is not drawn in Figure 1, because the elongation factors are preincubated with saturating amounts of aminoacyl-tRNA and GTP. Therefore, the initial burst of elongation has a rate which is independent of the rate of regeneration of ternary complex and reflects only the discharge of preformed ternary complexes. The second phase of poly(Phe) synthesis is apparently carried out in a steady-state under which the formation of ternary complex is rate limiting. The biphasic behaviour of the rate of poly(Phe) synthesis can be used to determine the concentration of active ternary complex initially present in the system. If we extrapolate the second steady-state course of the elongation to zero time, the intercept yields an approximate measure of the total amount of active EF-Tu in the system. This approximation is a good one since the time scales for the initial burst and the subsequent steady-state incorporation of phenylalanine are well separated in the absence of EF-Ts (Figure 1). The spontaneous rate v_{sp} at which peptide bonds are formed without EF-Ts, normalized to the concentration of active EF-Tu, was estimated from Figure 1 to be 0.015 s^{-1}. Thus, the turnover time for the EF-Tu cycle is close to 65 s in the absence of EF-Ts.

The effect of EF-Ts can be estimated by taking the difference between the EF-Tu normalized steady-state rate of elongation (v) in the presence of EF-Ts and the normalized spontaneous rate v_{sp} in its absence and expressing it according to

$$v - v_{sp} = \frac{[\text{EF-Ts}]_o \cdot k_{cat}}{[\text{EF-Tu}]_o + K_M} \quad (1)$$

Here $[\text{EF-Ts}]_o$ is the input concentration of EF-Ts and $[\text{EF-Tu}]_o$ is the input concentration of active EF-Tu. The formal parameters k_{cat} and K_M in formula (1) faithfully reflect the characteristics of the cycling of the EF-Ts under the condition that almost all active EF-Tu is in complex with GDP under the steady-state phase of poly(Phe) synthesis. This single condition summarizes the two requirements that EF-Ts is limiting the rate of the EF-Tu-cycle and that the EF-Tu-cycle is the rate-limiting step of poly(Phe) synthesis.

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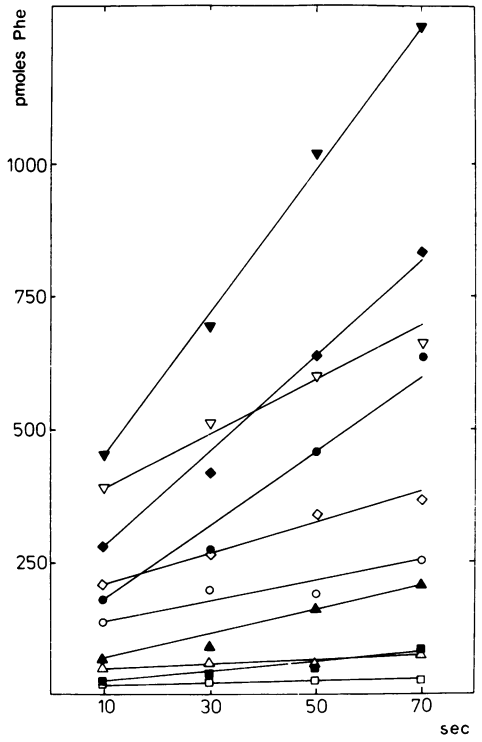

Fig. 1. Time course of poly(Phe) synthesis at five different concentrations of EF-Tu in the presence and absence of EF-Ts. Open symbols indicate the absence and filled symbols the presence of 5 nM EF-Ts. The estimated EF-Tu concentrations were 0.15 μM (□, ■), 0.40 μM (△, ▲), 1.20 μM (○, ●), 1.80 μM (◇, ◆) and 3.40 μM (▽, ▼). Experiments were performed as described in **Materials and methods**. Assays for different time points were conducted in separate tubes. The reactions were quenched after the indicated time, processed and calculated as described by Jelenc and Kurland (1979).

In Figure 2 we have plotted $[EF\text{-}Ts]_o/(v - v_{sp})$ as a function of $[EF\text{-}Tu]_o$. The slope of the straight line thus gives an estimate of $1/k_{cat}$, and the intercept at the ordinate gives an estimate of K_M/k_{cat}. The k_{cat} value for the factor interaction according to Figure 2 is 32 s^{-1} while the K_M value is 2.9×10^{-6} M. Thus, our data indicate that EF-Ts has a cycle time close to 30 ms during polypeptide synthesis.

Nucleotide exchange assay

If the rate-limiting step in poly(Phe) synthesis under the experimental conditions of the preceding section is the release of GDP from the EF-Tu complex, the direct measurement of this dissociation as well as the catalytic effect of EF-Ts on the reaction should have kinetic characteristics in common with those obtained in the translation assay. Therefore, we have measured the nucleotide exchange rate for EF-Tu in the presence and absence of EF-Ts in a system containing GTP and aminoacyl-tRNA.

The assay employed here consists of the pyruvate kinase dependent conversion of [^{3}H]GDP to [^{3}H]GTP in the incubation mixture and a subsequent measurement of the [^{3}H]GDP to [^{3}H]GTP ratio after t.l.c. This ratio is used as a measure of the ratio of EF-Tu·[^{3}H]GDP to released [^{3}H]GDP, which has been converted to [^{3}H]GTP. Control experiments have shown that EF-Ts alone cannot carry out this phosphorylation; that factor-bound GDP is not converted to GTP by the kinase; and that the conversion of free [^{3}H]GDP to [^{3}H]GTP is rapid in relation to its release from EF-Tu. Thus, the concentration of free [^{3}H]GDP can be neglected and the amount of [^{3}H]GDP measured at different time points, here called $[EF\text{-}Tu\cdot GDP]_t$, reflects the current amount of EF-Tu in complex

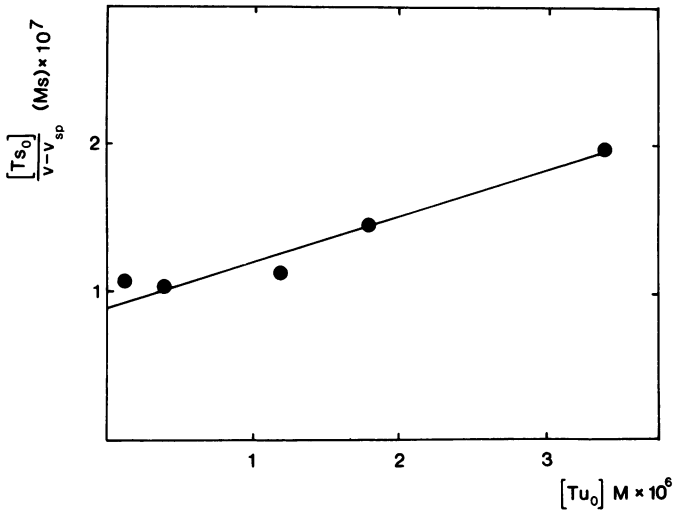

Fig. 2. Determination of k_{cat} and K_M for the interaction between EF-Ts and EF-Tu in poly(U)-primed peptide synthesis. The points in the figure are calculated as described in the text from the experimental data presented in Figure 1.

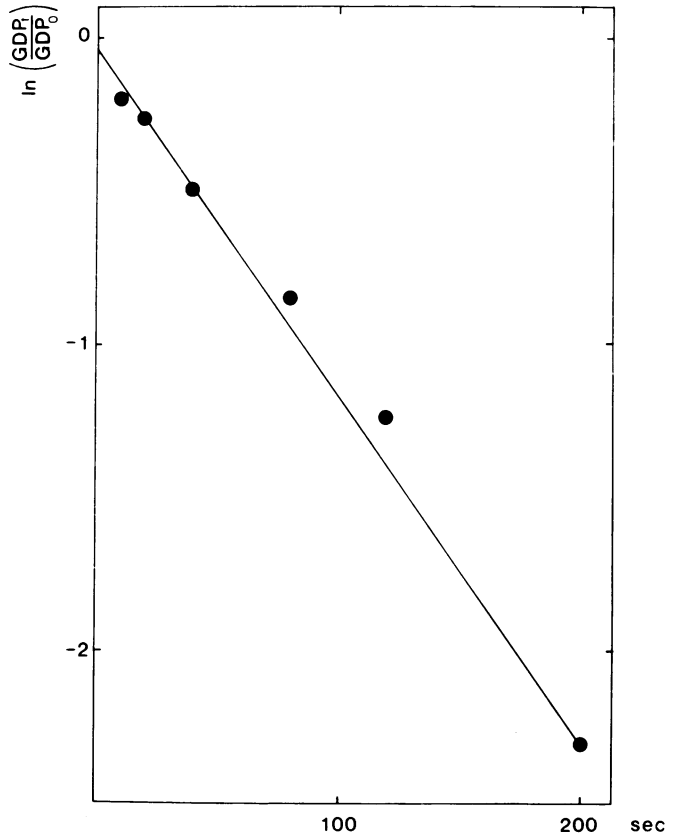

Fig. 3. Time course of dissociation of GDP from EF-Tu in the absence of EF-Ts. The experiment was conducted as described in **Materials and methods**: 20 μl samples were withdrawn at indicated times.

with GDP. In Figure 3 we have plotted

$$\frac{[EF\text{-}Tu\cdot GDP]_t}{[EF\text{-}Tu\cdot GDP]_o}$$

against time. In this semilogarithmic representation a straight line is obtained which indicates that the rate of decay of EF-Tu·GDP follows first-order kinetics characterized by a rate constant $k_{sp} = 0.011\ s^{-1}$.

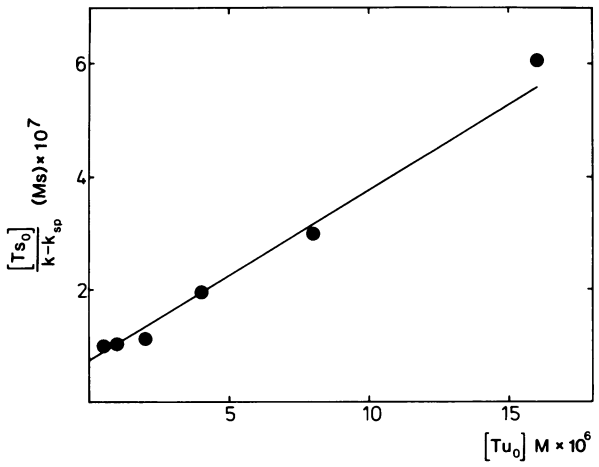

Fig. 4. Determination of k_{cat} and K_M for the interaction between EF-Ts and EF-Tu in the nucleotide exchange assay. The experiments were performed as described in **Materials and methods**, and the first-order rate constants were determined from a semi-logarithmic plot (see Figure 3) in the absence or presence of EF-Ts. For further details see text.

The corresponding value, v_{sp}, obtained in the translation system was 0.015. The difference between these two rates is 0.004 s^{-1}. This number is small but reproducible and could be related to a minor EF-Ts contaminant in our elongation factor G (EF-G) preparations. Indeed, if EF-G is present in the nucleotide exchange assay at the same concentration as used in the poly(Phe)-synthesis system the two rates k_{sp} and v_{sp} become virtually identical (data not shown).

The effect of EF-Ts on the exchange reaction can be determined by measuring the rate of release of [^{3}H]GDP in the presence of EF-Ts at different concentrations of EF-Tu. The experiments were carried out at EF-Ts concentrations sufficiently high that the rate of release of [^{3}H]GDP from EF-Tu was significantly larger than the spontaneous release in the absence of EF-Ts. At the same time the release rate had to be kept within the limits of measurability, i.e., <95% of the [^{3}H]GDP released within 10 s.

The rate of decay of [^{3}H]GDP in the system apparently follows first-order kinetics and can be described by a single rate constant k. The parameters characterizing the factor interaction were evaluated from the relationship:

$$k - k_{sp} = \frac{[EF\text{-}Ts]_o\, k_{cat}}{[EF\text{-}Tu\cdot[^3H]GDP]_o + K_M} \qquad (2)$$

In Figure 4 $[ET\text{-}Ts]_o/(k - k_{sp})$ is displayed as a function of the zero-time concentration of EF-Tu in complex with GDP. The slope of this plot yields a value for k_{cat} of 32 s^{-1} (compared to 32 s^{-1} from the translation assay). The estimate of K_M from this plot is 2.4 x 10^{-6} M (compared to 2.9 x 10^{-6} M from the translation assay). Formula (2) is closely analogous to the steady-state relationship (1) and, not surprisingly, the two methods of obtaining the characteristics of the factor interaction give very similar results.

Discussion

The experiments described here show that it is possible to use a steady-state polypeptide synthesizing system to study the kinetics of the partial reactions carried out by individual elongation factors. In particular, we have shown that in our system the rate of release of GDP from the complex with EF-Tu is close to 0.015 s^{-1} in the absence of EF-Ts. We also show that this release step can be accelerated by EF-Ts to a rate close to 30 s^{-1}.

We have compared the kinetic characteristics of EF-Tu and EF-Ts in polypeptide synthesis with those manifest in an assay system measuring directly the release of GDP from EF-Tu·GDP complex. The rate of release of GDP in the absence of EF-Ts under our conditions is approximately 0.011 s^{-1}; this is comparable to 0.015 s^{-1} for the corresponding rate constant for EF-Tu in polypeptide synthesis. Furthermore, the interaction of EF-Ts with EF-Tu is characterized by a k_{cat} of 32 s^{-1} amd a K_M of 2.4 x 10^{-6} M in the nucleotide release assay. The corresponding parameters for the polypeptide assay are 32 s^{-1} and 2.9 x 10^{-6} M, respectively. The agreement between these values obtained in the two assay systems strongly supports the interpretation that the rate-limiting step of the EF-Tu cycle in the absence of EF-Ts is the release of GDP from the factor, and that the principal function of EF-Ts is to accelerate this dissociation step (Miller and Weissbach, 1977).

Recently, Chau *et al.* (1981) have studied the EF-Ts catalyzed release of GDP from EF-Tu. Their choice of assay, where [^{3}H]GDP on EF-Tu is replaced by cold GDP instead of GTP as well as charged tRNA as in protein synthesis, makes a direct comparison with our work difficult. In addition, they have worked at a lower temperature (21°C) and under buffer conditions known to be suboptimal for ribosomal translation (Jelenc and Kurland, 1979). They obtain a maximal rate of turnover for EF-Ts of ∼1000 s^{-1} which is 30-fold greater than the k_{cat} value for the factor interaction obtained in our system. One reasonable explanation for this discrepancy might be that GDP induces a release of EF-Ts from EF-Tu considerably faster than does GTP. If so their result is interesting in itself, but it may have little relevance to the EF-Tu cycle in living cells.

We have used the nucleotide exchange assay to make preliminary investigations of the influence of the concentrations of GTP and charged tRNA on the EF-Ts catalyzed release of GDP from EF-Tu. We find that the turnover rate of EF-Ts is accelerated by increasing concentrations of GTP up to ∼1 mM at a concentration of EF-Tu·GDP of 2 x 10^{-6} M. Further increase in the GTP concentration has no effect on the GDP dissociation rate under these conditions. Indeed, Fasano *et al.* (1978) have observed a remarkably small association rate constant for GTP binding to the binary complex EF-Tu·EF-Ts (k_a = 4.5 x 10^4 M^{-1} s^{-1}). Their result implies that, when this reaction step is part of the EF-Ts cycle, it might be the rate-limiting step for the factor interaction at GTP concentrations of <1 mM. Charged $tRNA^{Phe}$ at concentrations up to 3.5 x 10^{-6} M had no effect on the GDP release rate.

It is worth emphasizing that there is only a formal equivalence between equations (1) and (2), because they describe different conditions. The first describes a steady-state situation and the second a relaxation experiment. The validity of equation (1) rests on the assumption that a major fraction of the total, active EF-Tu exists as an EF-Tu·GDP complex. When this is so, both in the absence and in the presence of EF-Ts, then the differential $v - v_{sp}$ is a proper measure of the EF-Ts catalyzed pathway for the dissociation of GDP from EF-Tu.

Furthermore, equation (2) is strictly valid only when the dissociation of GDP follows first-order kinetics in the presence as well as in the absence of EF-Ts. This condition can be translated into the requirement that the concentration

of free EF-Ts stays constant during the time course of the GDP dissociation. Although no significant deviation from first-order kinetics was detected in the experiments, equation (2) must be regarded as an approximation.

Finally, it is possible to conclude from the *in vivo* concentration of EF-Ts, which can be estimated to be 3 x 10^{-5} M (Chau *et al.*, 1981), and the present estimate of the K_M value for the factor interaction (2.5 x 10^{-6} M) that the EF-Ts catalyzed release of GDP from EF-Tu in the bacterium proceeds at a rate near to 30 s^{-1}.

Materials and methods

Chemicals

All chemicals used were of the highest purity available. Poly(U), phenylalanine, phosphoenolpyruvate (PEP), GTP, putrescine, spermidine, myokinase (E.C.2.7.4.3) and pyruvate kinase (E.C.2.7.1.40) were purchased from Sigma. *Escherichia coli* tRNA from strain MRE 600 was obtained from Boehringer-Mannheim. Thin layer plates (PEI polygram CEL 300) came from Macerey-Nagel, Duren, GFR. Radioactive substrates, [^{3}H]GDP and [^{3}H]phenylalanine, were purchased from the Radiochemical Centre, Amersham, UK.

Buffer

Polymix (Jelenc, 1980; Jelenc and Kurland, 1979): 5 mM Mg^{2+}, 0.5 mM Ca^{2+}, 8 mM putrescine, 1 mM spermidine, 5 mM phosphate, 5 mM NH^+_4, 95 mM K^+, 1 mM dithioerythritol, pH 7.5. Working strength buffer was prepared by mixing, in the correct proportions, 100 mM potassium phosphate (pH 7.5) and a 10-fold concentrate without phosphate (pH 7.5) prepared according to the procedure described in Jelenc (1980).

Purifications

Ribosomes were prepared from frozen MRE 600 cells (Public Health Laboratory Service) as described by Jelenc (1980) and stored in buffer containing 30% v/v of methanol at −20°C as described in Jelenc (1980). EF-G was purified according to Wagner and Kurland (1980) and stored in polymix at −80°C. The purification of EF-Tu followed the method of Lebermann *et al.* (1980) with modifications described in Wagner *et al.* (1981). The factor was stored at −80°C in polymix. EF-Ts was obtained through the procedure of Arai *et al.* (1972) and stored in polymix at −80°C. The purification of phenylalanyl-tRNA synthetase (Phe S) followed the scheme described in Wagner *et al.* (1981). The enzyme was stored in polymix buffer containing 25% glycerol at −20°C.

Preparation of EF-Tu·[^{3}H]GDP

Purified EF-Tu·GDP together with radioactive [^{3}H]GDP was dialyzed against 100 volumes of polymix buffer containing 10 μM GDP until the radioactivity outside the bag reached a plateau. This procedure took 10−15 h. The specific activity obtained was ∿40 c.p.m./pmol EF-Tu·GDP when counted in Rotiszint scintillation fluid (Lab Kemi, Frölunda, Sweden) for total tritium counts. The preparation was kept at −80°C until it was used.

Nucleotide exchange

The conventional assay for EF-Tu-bound nucleotide involves trapping the factor complex on nitrocellulose filters after the reaction has been stopped by a dilution into buffer (Miller and Weissbach, 1970b). We have, instead, used an assay in which radioactive GDP released from EF-Tu is enzymatically converted into radioactive GTP and the ratio of the two nucleotides is measured to determine the relative amounts of bound and unbound nucleotide in the incubation mixture.

A standard assay mixture contains, in polymix, 4 mg/ml bulk tRNA, 50 μM phenylalanine, 50 units/ml myokinase, 20 units/ml Phe S, 1 mM GTP, 1 mM ATP, 6 mM PEP, 10 μg/ml pyruvate kinase, 0−20 nM EF-Ts and 0−16 μM EF-Tu. This mixture was preincubated simultaneously with, but separately from, the EF-Tu·[^{3}H]GDP mixture in polymix described above, for 5 min at 37°C. Then 180 μl of the unlabelled mixture and 20 μl of the labelled mixture were mixed at 37°C. Samples of 20 μl were withdrawn at 10−20 s intervals and mixed with 10 μl of 1.2 M $HClO_4$ solution to stop the reaction. The samples were treated as described by Bagnara and Finch (1972) and then fractionated on thin layer plates (see above) developed in 0.5 M potassium phosphate pH 3.5. The nucleotide spots were localized on the dried plates, and cut out. The samples were then suspended in a 0.5% (w/v) PPO toluene solution and radioactivity was measured in a scintillation counter.

Translation assay

The steady state cycle time of EF-Tu was determined in a poly(U)-primed protein synthesis system, working close to the elongation rate *in vivo* (Wagner *et al.*, 1981). The experiments were designed such that the EF-Tu concentration was rate limiting over a wide range of EF-Ts concentrations.

The assay consists of two mixtures that are prepared on ice. Both of them contain, in addition to the polymix buffer components (see above), 1 mM ATP, 1 mM GTP, 6 mM PEP, 10 μg/ml pyruvate kinase and 10 units/ml myokinase. In addition, mixture I contains in 50 μl: 200 pmol of ribosomes, 27 μg poly(U), 330 μg of total *E. coli* tRNA. Mixture II contains in 50 μl: 5000 pmol [^{14}C]phenylalanine (4.7 c.p.m./pmol), 830 μg of total *E. coli* tRNA, 200 pmol of EF-G, 0−340 pmol of EF-Tu, 5 pmol of EF-Ts and ∿30 units of Phe S.

Mixtures I and II are preincubated separately for 12 min at 37°C. The elongation reaction is started by pipetting 50 μl of mixture I into 50 μl of mixture II with a prewarmed pipette tip. The assay is stopped at 10, 30, 50 or 70 s by addition of 3 ml trichloroacetic acid containing 0.5 w/v of phenylalanine. The background value was obtained from an assay performed in the absence of ribosomes. Filtrations and calculations are carried out as described by Jelenc and Kurland (1979).

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