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Late events of translation initiation in bacteria: a kinetic analysis

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Binding of the 50S ribosomal subunit to the 30S initiation complex and the subsequent transition from the initiation to the elongation phase up to the synthesis of the first peptide bond represent crucial steps in the translation pathway. The reactions that characterize these transitions were analyzed by quench-flow and fluorescence stopped-flow kinetic techniques. IF2-dependent GTP hydrolysis was fast (30/s) followed by slow P_i release from the complex (1.5/s). The latter step was rate limiting for subsequent A-site binding of EF-Tu·GTP·Phe-tRNA^{Phe} ternary complex. Most of the elemental rate constants of A-site binding were similar to those measured on poly(U), with the notable exception of the formation of the first peptide bond which occurred at a rate of 0.2/s. Omission of GTP or its replacement with GDP had no effect, indicating that neither the adjustment of fMet-tRNA^{fMet} in the P site nor the release of IF2 from the ribosome required GTP hydrolysis.

Keywords: A-site binding/fast kinetics/IF2-dependent GTPase/phosphate release/translation initiation

Introduction

Initiation of protein synthesis in bacteria involves several steps that ultimately bring together the 70S ribosome, the initiator fMet-tRNA^{fMet} and the translation initiation region of the mRNA to form a functional complex, ready to enter the elongation phase of translation. During the transition from the 30S to the 70S initiation complex and the subsequent passage from the initiation to the elongation phase of translation, initiation factors IF1 and IF3 are ejected from the ribosome, IF2 hydrolyzes GTP and dissociates from the ribosome, the acceptor end of initiator fMet-tRNA^{fMet} becomes substrate for the ribosomal peptidyltransferase and the first dipeptide is formed (for reviews, see Hershey, 1987; Gualerzi and Pon, 1990; Gualerzi *et al*., 2000).

Among the three factors (IF1, IF2 and IF3) that kinetically govern initiation, IF2 is the one that assists the initiation process throughout most of the steps. IF2 binds to the 30S subunit and promotes first the codon-independent and then the initiation codon-dependent binding of fMet-tRNA^{fMet} (Wintermeyer and Gualerzi, 1983; Canonaco *et al*., 1986; Gualerzi and Wintermeyer, 1986; La Teana *et al*., 1996); subsequently, IF2 stimulates the association of 30S and 50S subunits (Grunberg-Manago *et al*., 1975) and finally allows the adjustment of fMet-tRNA^{fMet} in the bona fide P-site where it can form the first peptide bond with the aminoacyl-tRNA (aa-tRNA) bound to the second codon triplet and brought to the A-site by EF-Tu·GTP (La Teana *et al*., 1996).

As in the case of the translation factors EF-Tu, EF-G and RF3, IF2 is a GTPase that is activated by the interaction with the 50S ribosomal subunit. All translation factors appear to bind to overlapping sites on the ribosome. This overlap is the probable basis for the mutual incompatibility between the binding of EF-Tu and EF-G and for a similar incompatibility believed to exist between IF2 and the two elongation factors (Parmeggiani and Sander, 1981; Luchin *et al*., 1999, and references cited therein).

Stimulation by IF2 of fMet-tRNA^{fMet} binding to the ribosomes is accomplished by an increase in the rate of codon–anticodon interaction and by a decrease in the dissociation rate of the ribosome–tRNA complex. Since the affinity of IF2 for aa-tRNAs bearing a blocked α-amino group is at least two orders of magnitude greater than that displayed for non-modified aa-tRNAs, IF2 strongly favors, both *in vitro* and *in vivo*, binding of fMet-tRNA^{fMet} to the ribosome. In contrast to EF-Tu, whose interaction with aa-tRNAs depends on the presence of GTP, the interaction of IF2 with fMet-tRNA^{fMet} is not affected by nucleotides (Petersen *et al*., 1979); only the affinity of IF2 for the 30S subunit is somewhat influenced by the nature of the guanosine nucleotide bound to the factor (Pon *et al*., 1985). Nonetheless, since the cellular concentration of GTP is at least one order of magnitude higher than the K_d of the IF2·GTP complex, IF2 is likely to perform all of its functions on the 30S subunit in the GTP-bound state. The GTP molecule is hydrolyzed only during formation of the 70S initiation complex. Although the release of IF2 from the 70S initiation complex (Hershey, 1987; Luchin *et al*., 1999) and/or the adjustment of the initiator tRNA in the P-site (La Teana *et al*., 1996, and references therein) are thought to require (or, at least, to be accelerated by) GTP hydrolysis, the role of the IF2-dependent GTPase remained elusive.

Here, the kinetics of IF2-dependent GTP hydrolysis and of the steps that mark the transition from the initiation to the elongation phase of translation were studied by quench-flow and fluorescence stopped-flow techniques. Using ribosomes programmed with a model mRNA (022mRNA; La Teana *et al*., 1993) with AUG and UUU as first and second codon, respectively, we investigated the kinetics of the following steps: (i) IF2-dependent GTPase; (ii) P_i release; (iii) binding of ternary complex EF-

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Tu·GTP·Phe-tRNA^{Phe} to 70S initiation complexes; and (iv) the formation of the first dipeptide, fMetPhe. The last two steps are used as indicator reactions of the transition of the 70S initiation complex to its elongation-competent state. A kinetic mechanism for the transition from the initiation to the elongation phase of protein synthesis is derived from the data.

Results

Rapid kinetics of IF2-dependent GTP hydrolysis

GTP hydrolysis during 70S initiation complex formation was studied by quench-flow measurements. Two solutions, one containing 30S subunits, 022mRNA, IF1, IF2, IF3, f[^{3}H]Met-tRNA^{fMet} and [γ-^{32}P]GTP, and another containing 50S subunits, were mixed rapidly and the time course of GTP hydrolysis was measured. Rapid hydrolysis of GTP was observed with an apparent rate constant of k_{app} = 30 ± 5/s, reflecting single-round GTP hydrolysis by IF2 after joining of 50S to the 30S initiation complex (Figure 1A). Subsequent slow multiple turnover GTP hydrolysis was observed due to the uncoupled GTP hydrolysis by IF2 on the ribosomes; the rate constant of the multiple turnover reaction was 0.15 ± 0.02/s (not shown).

GTP hydrolysis by IF2 requires binding of 50S to the 30S initiation complex; if the latter reaction limited the rate of GTP hydrolysis, there should be a delay in the time course of GTP cleavage. Figure 1A shows that the reaction progressed in a clearly exponential fashion, and that there is no significant delay phase. Furthermore, the rate of GTP hydrolysis by IF2 did not change upon addition of increasing concentrations of 50S subunits (Figure 1B). Thus, joining of 50S subunits to the 30S initiation complex occurred much more rapidly than the subsequent hydrolysis of GTP.

P_i release from IF2

P_i release was studied according to the method of Brune *et al*. (1994), using a fluorescent derivative of phosphate-binding protein (PBP) from *Escherichia coli*, PBP-MDCC. Binding of P_i to MDCC-labeled PBP is rapid (k_{on} = 10^8/M/s) and tight (K_d = 0.1 μM), and the formation of the complex strongly increases the fluorescence of MDCC (Brune *et al*., 1994). After a short lag phase (~200 ms), which included the time required for GTP hydrolysis and possibly other steps, a rapid increase in fluorescence was observed, reflecting P_i dissociation after the first round of GTP hydrolysis, followed by a further slower increase due to multiple rounds of GTP hydrolysis (Figure 2A). In the first round, P_i was released at a rate of 1.5 ± 0.5/s, independently of the concentration of 50S subunits (Figure 2B), suggesting that this value represented the rate constant of P_i release (Scheme 1). The rate of the turnover reaction (0.15 ± 0.05/s) was the same as measured in the GTPase reaction (not shown). The molecular basis for the 200 ms delay is not known, because only ~30 ms are required to complete the GTPase reaction ($1/k_{GTPase}$ = 1/30 s).

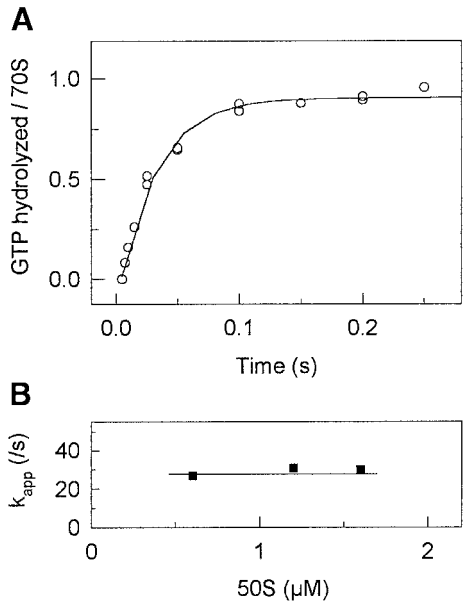


Fig. 1. Single round of IF2-dependent GTP hydrolysis. (**A**) Time course of GTP hydrolysis in the presence of 0.6 μM 50S. The data were fitted by a single exponential function with k_{app} = 30 ± 5/s. (**B**) Dependence of k_{app} upon 50S subunit concentration.

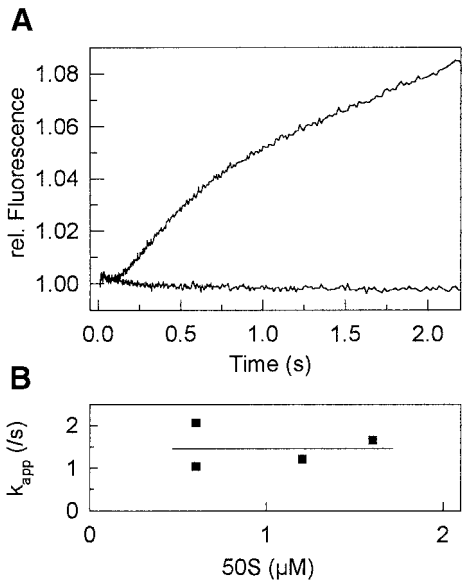
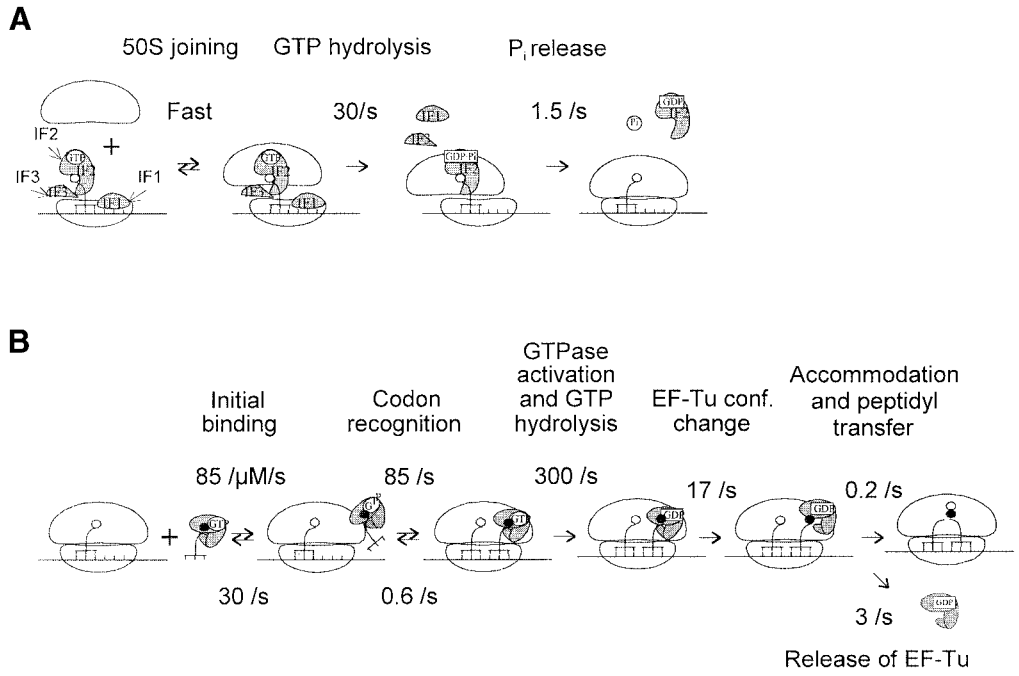


Fig. 2. P_i release following GTP hydrolysis in IF2. (**A**) Time course of P_i release in the presence of 0.6 μM 50S subunits. The data were fitted by a function comprising the sum of two exponential terms [delay and k_{app}(fast)] and a linear term [k_{app}(slow)]. The parameters of the fit: k_{app}(fast) = 1.5 ± 0.5/s, k_{app}(slow) = 0.15 ± 0.10/s. Lower curve, control. (**B**) Dependence of k_{app}(fast) on 50S subunit concentration.

Binding of the ternary complex EF-Tu·GTP·Phe-tRNA^{Phe} to the 70S initiation complex

If IF2-dependent GTP hydrolysis is required for the release of IF2 from the ribosome upon formation of the 70S initiation complex, it is possible that the presence of IF2 on the ribosome, or its slow release, may interfere with the interaction of ternary complex with the A-site. On the other hand, a failure of IF2 to place the initiator fMet-tRNA^{fMet} correctly into the P-site in the absence of GTP should result in a reduced efficiency and/or a slower formation of dipeptide (i.e. fMetPhe in the case of



Scheme 1. Transition from initiation to elongation. (**A**) Kinetic mechanism of transition from the 30S to 70S initiation complex. (**B**) EF-Tu-dependent binding of aa-tRNA to the ribosomal A-site.

022mRNA). Thus, the ability of the 70S initiation complex to bind EF-Tu·GTP·Phe-tRNA^{Phe} was used to study the completion of initiation and the transition to the elongation state of the ribosome. The 30S initiation complex was formed in the presence of GTP or GDP, or in the absence of nucleotide. The ternary complex EF-Tu·GTP·Phe-tRNA^{Phe} was purified from unbound GTP by gel filtration (Rodnina *et al*., 1994a). The extent of ternary complex binding was measured by nitrocellulose filtration, and the kinetics by stopped-flow, monitoring the fluorescence change of proflavin attached to the D loop of tRNA^{Phe} (Rodnina *et al*., 1994a).

The multistep process of aa-tRNA binding to the A-site of the ribosome is depicted in Scheme 1. In the first step, a ternary complex of aa-tRNA with elongation factor Tu (EF-Tu) and GTP forms a labile complex with the ribosome (initial binding, rate constants of the forward and backward reactions are defined as k_1 and k_{-1}, respectively) (Rodnina *et al*., 1996). Subsequent codon recognition (k_2 and k_{-2}) triggers GTP hydrolysis (k_3) (Rodnina *et al*., 1995) which results in a large-scale conformational change of EF-Tu to the GDP-bound form (k_4) (Dell *et al*., 1990; Abel *et al*., 1996; Polekhina *et al*., 1996). As a consequence, aa-tRNA is released from EF-Tu and enters the A-site on the 50S ribosomal subunit (accommodation) to take part in peptide bond formation (k_5), whereas EF-Tu·GDP dissociates from the ribosome (k_6) (Rodnina *et al*., 1995).

In the stopped-flow experiment, the 30S initiation complex was pre-mixed with 50S subunits in the pre-mixing chamber of the stopped-flow apparatus to form the 70S initiation complex which was then mixed rapidly with ternary complex in the second mixing chamber, and the proflavin fluorescence was monitored. As shown in Figure 3A, the signal changed in a biphasic way: a rapid increase in the fluorescence was followed by a slower decrease. Qualitatively, the same result had been observed previously with poly(U)-programmed ribosomes (Rodnina *et al*., 1994a); therefore, the assignment of the respective fluorescence changes to particular steps was taken from the poly(U) system (Pape *et al*., 1998, and references cited therein). The increase in proflavin fluorescence reflects the steps of initial codon-independent binding of the ternary complex to the ribosome as well as the codon recognition (Scheme 1), as established in experiments with non-programmed ribosomes (Rodnina *et al*., 1996) and with poly(U)-programmed ribosomes but in the presence of a non-hydrolyzable GTP analog in the ternary complex (Rodnina *et al*., 1994a). Further steps of EF-Tu activation for the GTPase reaction and GTP hydrolysis do not change the fluorescence of proflavin. The fluorescence decrease reflects the coupled conformational change of EF-Tu and aa-tRNA during the transition of the factor from the GTP to the GDP form and aa-tRNA release from EF-Tu. In the presence of poly(U) and AcPhe-tRNA^{Phe} in the P-site, this step is partly masked by the increase of fluorescence of the A-site-bound tRNA due to its accommodation and peptide bond formation (Rodnina *et al*., 1994a; Pape *et al*., 1998); in the mRNA system, the latter two reactions do not change proflavin fluorescence (Figure 3A). Quantitative differences between the mRNA and poly(U) systems in both apparent rate constants and amplitudes are attributed to the different buffer conditions (Pape *et al*., 1998), and to the presence of a different tRNA in the P-site (Rodnina *et al*., 1994a).

To study the effect of the nucleotide in IF2 on the transition from the initiation to the elongation step, experiments were performed in the presence of GTP or GDP, or without nucleotide during initiation. The presence or the nature of the nucleotide did not significantly influence the extent of f[^{3}H]Met-tRNA^{fMet} binding to the 30S subunits (Pon *et al*., 1985; La Teana *et al*., 1996; and

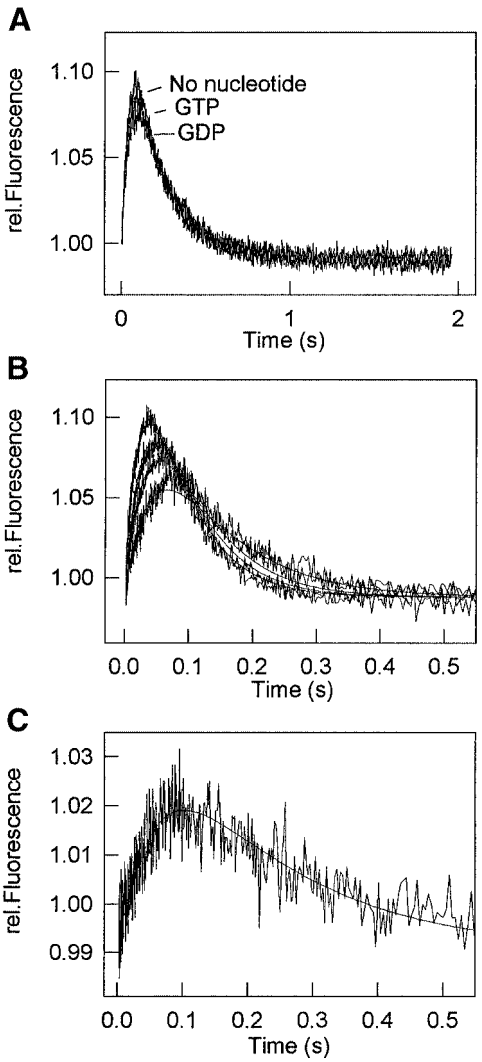


Fig. 3. Binding of ternary complex to the 70S initiation complex. (**A**) Time course of EF-Tu·GTP·[^{14}C]Phe-tRNA^{Phe} binding in the presence of GTP or GDP, or in the absence of nucleotides. According to the nitrocellulose filtration, binding of [^{14}C]Phe-tRNA^{Phe} to the ribosome was 98, 95 and 87% of the added complex in the presence of GTP and GDP, or in the absence of nucleotide, respectively. (**B**) Time courses of ternary complex binding to 0.3 (lowest curve), 0.5, 0.7 and 1.0 μM (highest curve) 70S initiation complexes monitored by fluorescence changes of Prf16/17 in tRNA. (**C**) Time course of ternary complex binding to 0.7 μM 70S initiation complexes monitored by fluorescence changes of mant-GTP. Experiments were also performed at 0.3, 0.5 and 1.0 μM ribosomes (not shown).

data not shown). The time course of ternary complex binding to the ribosomes monitored by proflavin fluorescence was not affected by the presence or absence of nucleotide during formation of the initiation complex (Figure 3A). Also, the efficiency of ternary complex binding measured by nitrocellulose filtration did not depend on the presence of GTP (not shown). Thus, the 70S initiation complexes formed with or without GTP did not differ with respect to the binding of the ternary complex.

To determine the rate constants of the A-site binding to the first elongation codon, experiments were performed as described before (Pape *et al.*, 1998). In brief, the time course of A-site binding was measured at different concentrations of 70S initiation complex. Fluorescence

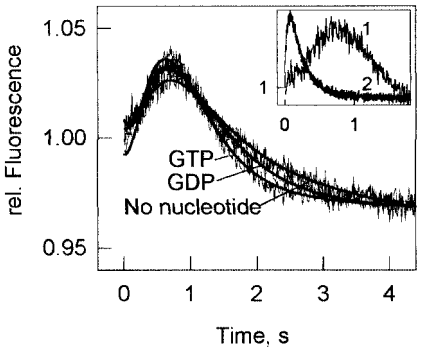


Fig. 4. Transition from initiation to elongation monitored by ternary complex binding. The 30S initiation complexes were prepared in the presence of GTP or GDP, or in the absence of nucleotide. Smooth lines show time curves simulated according to Scheme 1 using the following elemental rate constants: k_{GTP} = 30/s, k_{Pi} = 1.5/s, k_1 = 85/s, k_{-1} = 25/s, k_2 = 85/s, k_3 = 290/s, k_4= 17/s, k_5 = 0.2/s (c.f. Figures 1 and 2 and Table I). The data obtained in the presence of GDP or in the absence of the nucleotide were fitted by numerical integration using a similar model, except that the first two steps (k_{GTP} and k_{Pi}) were replaced by a single step; the value of the respective rate constant, $k_{transition}$, was determined to be 1.0 ± 0.5/s. The inset illustrates the delay phase of the ternary complex binding (curve 1) compared with the rapid interaction with the pre-formed 70S initiation complex (curve 2, from Figure 3A); both with GTP.

changes of proflavin in position 16/17 of the tRNA were used to monitor the conformational changes during initial binding of the EF-Tu ternary complex, codon recognition and release of aa-tRNA from EF-Tu followed by accommodation in the A-site (Figure 3B). Fluorescence changes of the GTP derivative, mant-GTP, were used to monitor the GTPase activation of EF-Tu and the dissociation of EF-Tu·mant-GDP from the ribosome (Figure 3C). The rate constants of initial binding of the ternary complex to the ribosome prior to codon recognition were determined separately from the ribosome titration experiments analogous to those shown in Figure 3B using non-programmed ribosomes (not shown). The value for the rate constant of dipeptide formation was taken from Figure 5A (see below). Elemental rate constants of A-site binding were determined by numerical integration using all available data (see Materials and methods). The results of the calculations are shown in Table I. The values of the elemental rate constants of A-site binding on 022mRNA were similar to those found with poly(U)-programmed ribosomes with two exceptions. For the rate constants k_1, k_{-1}, k_2, k_3 and k_6, the differences from the previously reported values (Pape *et al.*, 1998) were well within the range expected for the present ionic conditions (7 mM Mg^{2+}). Two rate constants, k_4 and, especially, k_5, were lower than in the poly(U) system.

Transition from initiation to elongation

To study the transition from the 30S initiation complex to the 70S initiation complex competent in ternary complex binding, stopped-flow experiments were performed using a different mode of pre-mixing than that of Figure 3A. The 30S initiation complexes were mixed rapidly with 50S subunits and ternary complex without prior incubation, and the proflavin fluorescence was monitored. In this case, the time course of ternary complex binding showed a clear delay phase (Figure 4). The duration of the delay was not

Table I. Elemental rate constants of EF-Tu-dependent aa-tRNA binding to the A site of 70S initiation complexes

Step	Rate constant (/s)	
Initial binding	k_1	85 ± 15[a]
	k_{-1}	30 ± 10[b]
Codon recognition	k_2	85 ± 10
	k_{-2}	0.6[c]
GTPase activation and GTP hydrolysis[d]	k_3	300 ± 100
GTP–GDP conformation change of EF-Tu	k_4	17 ± 5
Aa-tRNA accommodation and peptide bond formation[d]	k_5	0.2 ± 0.1
Dissociation of EF-Tu	k_6	3 ± 1

[a]/μM/s.
[b]Determined independently.
[c]Calculated by extrapolation of previously reported data (Pape *et al*., 1998).
[d]Grouped for the analysis, because the former reaction is rate limiting and the latter follows instantaneously.

affected by the nucleotide present during the 30S initiation step.

The data were evaluated quantitatively by numerical integration (Materials and methods). For the fitting, the model presented below (Scheme 1A) was used. In the first step, the 30S initiation complex binds the 50S subunit. As shown above, this step was fast compared with the subsequent reactions and was therefore not considered in the kinetic model (Materials and methods). The next step of GTP hydrolysis by IF2 proceeded with a rate constant of 30/s (cf. Figure 1) followed by P_i release, 1.5/s. When GDP or no nucleotide was present during the formation of the 30S initiation complex, these two steps were omitted from the calculations. Instead, to account for the delay in the ternary complex binding, a step was introduced which may include the dissociation of the initiation factors, final positioning of fMet-tRNA^{fMet} in the P-site or some other as yet unidentified reactions; kinetically, all these presumed processes were grouped into a single step limiting the rate of ternary complex binding. After the completion of the transition from the initiation to the elongation, the binding of the ternary complex took place (Scheme 1B). To describe the A-site binding reactions, the values for the rate constants for the present ionic conditions were used (Table I).

The results of fitting are shown as smooth lines in Figure 4. Scheme 1 fully accounts for the observed kinetic behavior in the presence of GTP, P_i release from IF2 being the rate-limiting step for the subsequent steps of A-site binding. The fitting yields a unique solution for the rate constant of the rate-limiting step completing the initiation in the presence of GDP or in the absence of the nucleotide. At ~1 ± 0.5/s, this value is very similar in the presence of GTP or GDP, or in the absence of the nucleotide.

Dipeptide formation

To test whether GTP hydrolysis is required for proper positioning of fMet-tRNA^{fMet} in the P-site, we analyzed the formation of the dipeptide fMetPhe after rapid mixing of 30S initiation complex and ternary complex with 50S subunits. The time course of dipeptide formation measured by quench-flow is shown in Figure 5. The rate of dipeptide formation was similar in the presence of GTP and GDP,

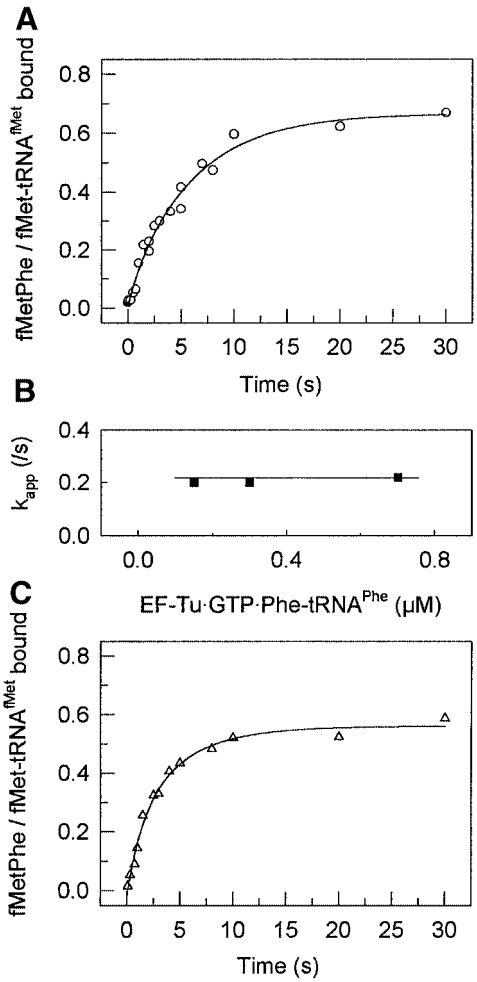


Fig. 5. Kinetics of fMetPhe formation. (**A**) Time course of dipeptide formation in the presence of GTP in the 30S initiation complex. The value of the apparent rate constant was determined by numerical integration (Materials and methods). (**B**) Dependence of k_{app} upon 50S subunit concentration. (**C**) Time course of dipeptide formation in the presence of GDP in the 30S initiation complex.

0.20 ± 0.04/s (Figure 5A) and 0.18 ± 0.03/s (Figure 5C), respectively, and did not increase further upon increasing the concentration of ternary complex (Figure 5B). The rate of fMetPhe formation was 35-fold lower than that of AcPhePhe formation under similar ionic conditions (Pape *et al*., 1998), but still compatible with the 0.3/s rate of initiation measured *in vivo* (Kennell and Riezman, 1977).

Dipeptide formation started after a lag period of ~750 ms, the time required for the transition from initiation to elongation and for the binding of the ternary complex. Replacement of GTP by GDP during the 30S initiation complex formation did not affect the duration of this lag significantly, indicating that GTP hydrolysis by IF2 did not accelerate the steps that led to production of the transpeptidation-competent complex. These results, together with the finding that there is no influence of the presence of GTP on the rate of dipeptide formation obtained with a standard experimental approach by La Teana *et al*. (1996), contradict the premise that GTP hydrolysis by IF2 is required for the adjustment of the fMet-tRNA in the P-site and for the formation of the first peptide bond (for references, see La Teana *et al*., 1996).

Discussion

Kinetic mechanism of transition from initiation to elongation

The multistep pathway of 30S initiation complex formation has been studied extensively (Gualerzi and Pon, 1990; Gualerzi *et al*., 2000). On the other hand, comparatively little is known concerning the later steps of initiation that characterize the transitions from the 30S to the 70S initiation complex and from the initiation to the elongation phase of protein synthesis. These transitions are of crucial importance insofar as they entail essential events such as the ejection of the initiation factors IF1 and IF3, the triggering of the GTPase activity of IF2, the adjustment of the fMet-tRNA^{fMet} in the P-site, the release of IF2 from the ribosome and the formation of the first peptide bond (initiation dipeptide) between fMet-tRNA^{fMet} and the aa-tRNA brought to the A-site by EF-Tu·GTP in response to the second codon of the mRNA.

The present results provide the first description of these events based on a rapid kinetic analysis of individual steps, which are the IF2-dependent hydrolysis of GTP, the release of P_i from the complex, the entry of the EF-Tu·GTP·aa-tRNA complex into the A-site of the 70S initiation complex and the formation of the first peptide bond (Scheme 1). Our results show that GTP is hydrolyzed rapidly by IF2 on the ribosome, triggered by the addition of 50S subunits to the 30S initiation complex containing IF2. The reaction has a rate constant of ~30/s and the time course of the GTP hydrolysis appears to follow a single exponential, with no lag in the onset of the reaction observed in the beginning. This means that after the mixing with the 30S initiation complex, the 50S subunit associates very rapidly to form a 70S complex and this is followed by fast GTP hydrolysis by IF2. The kinetics of P_i release from the complex, which occurs following an ~200 ms delay phase, are slower (1.5/s) than GTP hydrolysis, and GTP hydrolysis accounts for only ~30 ms (1/30/s) of the 200 ms lag phase of P_i release, indicating that IF2 stays in the GDP·P_i form for the rest of the delay time. Most probably, during this time, the factor remains bound to the ribosome at a site overlapping the binding site for EF-Tu·GTP·aa-tRNA, as during the same period of time the binding of the ternary complex is inhibited.

The binding of the EF-Tu·GTP·aa-tRNA complex is also delayed in the absence of nucleotide, or in the presence of GDP in initiation, as the rate constant of the rate-limiting step that determines the onset of the A-site binding in this situation, ~1/s, is similar to that observed in the presence of GTP, 1.5/s. This indicates that there is a conformational rearrangement that takes place after joining of the 50S subunit with the 30S initiation complex and that determines the rate of transition to the state which is competent for binding of the ternary complex. IF2 is expelled from the 70S ribosome only after this transition has taken place, regardless of whether it has GDP·P_i, GDP or no nucleotide bound. The same transition seems to limit the rate of P_i release from IF2.

The elemental rate constants of EF-Tu·GTP·Phe-tRNA^{Phe} binding to the 70S initiation complex up to the GTP hydrolysis step were found to be essentially the same as estimated for the poly(U) system at 7 mM Mg^{2+} in the absence of initiation factors (Pape *et al*., 1998). From the Mg^{2+} dependence of the rate constants measured by stopped-flow and quench-flow techniques at 5 and 10 mM Mg^{2+}, the following values are estimated for 7 mM Mg^{2+}: k_1 = 85 /μM/s, k_{-1} = 30/s, k_2 = 90/s and k_3 = 250/s; these values are almost identical to the values measured in the present study (Table I). Rate constants of steps following GTP hydrolysis are different. The present value of k_4 (17/s), the rate constant of the conformational change of EF-Tu, is three times lower than the (Mg^{2+}-independent) value measured for poly(U)-programmed ribosomes (60/s; Pape *et al*., 1998). While this difference may reflect another step where the ribosome has to adapt to elongation, it has to be interpreted with caution, because similar differences in k_4 were observed previously, for instance when different tRNAs were present in the *P*-site (Rodnina *et al*., 1994a, 1995), or when paromomycin was bound to the decoding center (Pape *et al*., 2000). Thus, the transition of EF-Tu from the GTP to the GDP form seems to be influenced by the ribosome, indicating that the conformational change of EF-Tu is coupled to a rearrangement of the ribosome that may be affected in various ways.

A large difference is observed between the present value of k_5 (0.2/s), the rate constant of fMetPhe formation, and that of AcPhePhe formation in the poly(U) system (7/s; Pape *et al*., 1998). As the rate of peptide bond formation is determined by the rate of aa-tRNA accommodation in the A-site (Pape *et al*., 1998), the different values of k_5 probably mean that the accommodation of Phe-tRNA^{Phe} is slower in the initiation complex with fMet-tRNA^{fMet} in the P-site compared with the ribosome complex programmed with poly(U) and containing AcPhe-tRNA^{Phe} in the P-site. As there is evidence suggesting that the accommodation step is coupled to a structural transition of 16S rRNA (Pape *et al*., 2000), the present results indicate that the same, or a related, transition of 16S rRNA may be required for the ribosome to perform the transition from initiation to elongation.

Does IF2 function as a switch operated by GTP hydrolysis?

IF2 is a large multidomain GTPase that belongs to the same subfamily of G proteins as EF-Tu, EF-G and RF3 (Bourne *et al*., 1991). Generally, G proteins are active, i.e. they interact with their respective effectors, in the GTP-bound form and are inactivated by GTP hydrolysis whereby they are switched into the inactive GDP-bound form. Whereas many GTPases follow this switch mechanism, recent evidence suggests that the functional mechanism of at least one translation factor, EF-G, is different. EF-G promotes the translocation of tRNAs on the ribosome, and kinetic measurements show that translocation is accelerated by GTP hydrolysis and that GTP hydrolysis precedes rather than follows translocation, in contrast to what is expected for a switch mechanism (Rodnina *et al*., 1997).

The present data show that IF2 also does not follow a switch mechanism. First, the formation of the 30S initiation complex is independent of the nucleotide in IF2 (La Teana *et al*., 1996), indicating that there is little affinity difference between the GTP- and GDP-bound forms of the protein in regulating the binding to its target (Pon *et al*., 1985). Secondly, GTP is hydrolyzed immediately after joining the 50S subunit, which is expected to

provide the element(s) required for the GTPase activation in translation factors. Finally, the transition from initiation to elongation is not affected by the nucleotide in IF2. Hence, none of the functional steps of initiation are regulated by GTP binding or hydrolysis.

GTP hydrolysis in G proteins leads to conformation changes of the G domain, notably in the switch 1 and 2 regions that are important for the interactions with effectors of the GTPases (Wittinghofer, 1998). Structural rearrangements that are induced by the cleavage of the bond between β and γ phosphate are likely to occur stepwise, via an intermediate that retains both GDP and P_i. Until now, the kinetics of P_i release have been measured for a number of GTPases and ATPases, including Ras (Nixon *et al*., 1995), transducin (Ting and Ho, 1991), myosin (Brune *et al*., 1994; Lionne *et al*., 1995; White *et al*., 1997; Suzuki *et al*., 1998), kinesin (Gilbert *et al*., 1995; Moyer *et al*., 1998), dynamin (Vernon *et al*., 1999), tubulin (Vandecandelaere *et al*., 1999) and EF-G (Rodnina *et al*., 1999). Whereas P_i is released instantaneously after GTP hydrolysis by Ras, it has been shown for transducin, myosin, kinesin and dynamin that P_i release is slow compared with the hydrolysis of ATP, and the ADP·P_i form of the latter proteins is crucial for their function. Interestingly, a significant delay between GTP hydrolysis and release of P_i was also observed in EF-G (Rodnina *et al*., 1999; A.Savelsbergh, M.V.Rodnina and W.Wintermeyer, unpublished), supporting the notion that the functional cycle of the factor differs from that of conventional switch GTPases (Rodnina *et al*., 1997). With a long delay between GTP hydrolysis and P_i release, IF2 seems to be yet another G protein that does not follow the switch mechanism.

Is there a function for GTP hydrolysis in IF2?

Two main functions have been attributed to the GTPase activity of IF2: the adjustment of the initiator tRNA in the ribosomal P-site and the release of IF2 from the ribosome. Previous work, carried out primarily with *Bacillus stearothermophilus* components, has shown that the presence of GTP is not mandatory to place fMet-$tRNA^{fMet}$ into the puromycin-reactive site unless the initiation complex is formed under suboptimal conditions (e.g. when there is no proper codon for the initiator tRNA) (La Teana *et al*., 1996). Since these studies were carried out using standard techniques which allow the investigation in a time range which is at the borderline of the rates (≈3.2 s/initiation event) found *in vivo* by Kennell and Riezman (1977), the conclusion that IF2-dependent GTP hydrolysis is not required for the adjustment of the initiator tRNA in the P-site could be limited to those physiological situations characterized by similar initiation rates.

The second role attributed to IF2-dependent GTP hydrolysis is that of allowing the dissociation of IF2 from the ribosome at the end of the initiation phase. This view is supported by early data as well as by a recent study in which the dominant lethal effect of IF2 mutations impairing the GTPase activity was attributed to the failure of this factor to dissociate from the ribosomes (Luchin *et al*., 1999), thereby inhibiting the binding of other translation factors (EF-G, EF-Tu and possibly RF3) or their function, as all translational GTPases may require the same ribosomal contacts, possibly with protein L7/L12 (Savelsbergh *et al*., 2000), to elicit their GTPase activity. Indeed, early experiments have also shown that IF2 may prevent the interaction of EF-Tu with the ribosome in the absence of GTP hydrolysis (Lockwood *et al*., 1974; Beaudry *et al*., 1979). Recently, extending the 'molecular mimicry' hypothesis, it has been suggested that in addition to IF1 (Moazed *et al*., 1995), IF2 also (in a binary complex with IF1) interacts with the A-site, mimicking the structure of EF-G which in turn mimicks that of the EF-Tu·GTP·aa-tRNA complex (Brock *et al*., 1998). Thus, it is thought that IF2 and EF-Tu interfere in binding, so that failure of IF2 to be released from the ribosome should prevent the binding of the EF-Tu ternary complex to the A-site (Luchin *et al*., 1999).

Although we cannot exclude that under our *in vitro* assay conditions a certain step of initiation may have become so unphysiologically slow and rate-limiting as to render the effect of GTP hydrolysis on the physiological rate-limiting step no longer detectable, our data indicate that: (i) the subunits associate properly into the 70S initiation complex without GTP; (ii) the positioning of the P-site-bound fMet-$tRNA^{fMet}$ is correct with respect to the peptidyltransferase center in the absence of GTP in the initiation complex; and (iii) the relative positioning of the tRNAs in the P- and the A-sites is the same in the presence and absence of GTP. Furthermore, our data indicate that IF2-dependent GTPase activity is not implicated in the ejection of IF2 from the complete 70S initiation complex.

These conclusions are in obvious conflict with current hypotheses concerning the role of the IF2-dependent GTPase in translation initiation. A possible reason for this discrepancy is that many of the conclusions reported in the earlier literature were based on results obtained with non-hydrolyzable GTP analogs, such as GDPCP and GDPNP, used to assess the possible role of GTP hydrolysis in the processes under study. It has become clear, however, that these analogs impose different structural constraints on IF2 which lead to the loss of functions not necessarily related to the lack of GTP hydrolysis (La Teana *et al*., 1996). Concerning the conflict of our data with the more recent results of Luchin *et al*. (1999), we would like to stress that, although we have obtained similar dominant lethal mutants of IF2 from *B.stearothermophilus*, we were unable to confirm that mutants lacking the GTPase activity remain preferentially associated with the ribosomes. Instead, we show that there is no relationship between the dominant lethal phenotype and the residual GTPase activity of mutant IF2 (Gualerzi *et al*., 2000; J.Tomšic, A.Smorlesi, R.Spurio, C.L.Pon and C.O.Gualerzi, in preparation).

Materials and methods

Buffers and reagents

Buffer A: 25 mM Tris–HCl pH 7.5, 70 mM NH_4Cl, 30 mM KCl and 7 mM $MgCl_2$. Buffer B: 20 mM Tris–HCl pH 7.5, 0.1 mM EDTA, 5 mM 2-mercaptoethanol, 10% glycerol, 0.1 mM benzamide and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). GTP, GDP, GDPNP, dithiothreitol (DTT), pyruvate kinase and phosphoenolpyruvate were from Roche Diagnostics. The purity of GTP was analyzed by HPLC and was found to be >95%. Chemicals were of analytical grade and purchased from Merck; radioactive compounds were from ICN or Amersham. All kinetic experiments were performed at 20°C.

Ribosomes, factors, tRNAs and mRNA

Escherichia coli MRE600 ribosomal subunits, $tRNA^{Phe}$ and $tRNA^{fMet}$ were kindly provided by Y.P.Semenkov and S.V.Kirillov (Gatchina, Russia). Initiation factors were prepared essentially as described (Gualerzi *et al*., 1991), except that for IF2 the purification was modified in that IF2-containing fractions from phosphocellulose chromatography were purified further by FPLC on MonoS using a linear gradient from 50 to 400 mM NH_4Cl in buffer B. IF2-containing fractions were identified by SDS–PAGE, pooled and dialyzed against buffer B containing 200 mM NH_4Cl. The protein concentration was determined both colorimetrically and from its molar extinction coefficient at 280 nm (Hershey *et al*., 1977).

EF-Tu, fluorescent $tRNA^{Phe}$ (Prf16/17) and purified ternary complex, EF-Tu·GTP·[^{14}C]Phe-$tRNA^{Phe}$, were prepared as described (Rodnina *et al*., 1994a). f[^{3}H]Met-$tRNA^{fMet}$ was prepared as in Rodnina *et al*. (1994b).

The 022mRNA (120 nucleotides) (La Teana *et al*., 1993) contained an appropriately spaced four nucleotide Shine–Dalgarno sequence followed by an AUG initiation codon and UUU as a first elongation codon. The rest of the translation initiation region of this mRNA also resembled the consensus sequence of natural mRNAs (La Teana *et al*., 1993).

GTPase activity

The 30S initiation complexes were prepared in buffer A using 0.6 μM 30S subunits, 0.9 μM of each of the initiation factors and of f[^{3}H]Met-$tRNA^{fMet}$, 1.8 μM 022mRNA and 72 μM [γ-^{32}P]GTP (~1000 d.p.m./pmol). After incubation at 37°C for 15 min, the complexes were stored on ice. Quench-flow experiments were performed using a KinTek RQF-3 apparatus. The 30S initiation complexes (final concentration 0.3 μM) were mixed rapidly with 50S subunits (26 μl each) at varying concentrations, as indicated (Figure 1). The reactions were quenched by 1 M $HClO_4$ and 3 mM KH_2PO_4, and [^{32}P]phosphate was determined by molybdate extraction as described (Rodnina *et al*., 1999). Time courses were evaluated by fitting a single exponential function. Time constants were reproducible within 20%.

P_i release

P_i release was monitored by the fluorescence change of phosphate-binding protein labeled with MDCC (Brune *et al*., 1994) in an SX-18MV stopped-flow spectrometer (Applied Photophysics). The fluorescence of MDCC was excited at 425 nm and monitored after passing a KV450 filter (Schott). The 30S initiation complexes were prepared as described above, except that non-radioactive GTP was used. The concentrations after mixing of 60 μl each of 30S initiation complexes and 50S subunits were the same as in the GTPase experiment (see above). The rate constant of P_i binding to MDCC-PBP, k_1 = 96/μM/s, was determined in separate experiments. The concentration of MDCC-PBP (2.5 μM) was chosen such that the rate of P_i binding to MDCC-PBP ($k_1' = k_1 \cdot$[MDCC-PBP] = 240/s) was much faster than the rate of GTP hydrolysis in IF2 (30/s) and thus the uptake of P_i liberated from the factor by MDCC-PBP was practically instantaneous. To minimize phosphate contamination, all solutions and the stopped-flow apparatus were pre-incubated with 600 μM 7-methylguanosine and 0.3 U/ml purine nucleoside phosphorylase (Brune *et al*., 1994). Rate constants were determined by fitting a function that included the delay before the onset of P_i release, an exponential term for P_i release and a slope for the linear turnover P_i release. The calculations were performed using the TableCurve software (Jandel Scientific).

A-site binding

Fluorescence stopped-flow measurements were performed on an SFM-3 apparatus (Biologic) or on an SX-18MV spectrometer (Applied Photophysics) and the data evaluated as described previously (Rodnina *et al*., 1994a; Pape *et al*., 1998). The fluorescence of proflavin was excited at 436 nm and measured after passing a KV500 filter (Schott). The fluorescence of mant-GTP was excited at 360 nm and measured after passing a KV408 filter (Schott).

The experiments of Figure 3A were performed by rapidly mixing equal volumes (60 μl each) of the purified ternary complex containing fluorescent tRNA and the 70S initiation complex. Final concentrations of ternary complex, 50S subunits and 30S initiation complex were 0.1, 0.3 and 0.39 μM, respectively. From experiment to experiment, the reproducibility of the rate constants given was about ±10%, that of the amplitudes about ±15%; within one experiment, the reproducibility from shot to shot was ~5% for both parameters.

To determine independently the rate constants of initial binding of the ternary complex to the ribosome, time courses of EF-Tu·GTP·Phe-$tRNA^{Phe}$(Prf16/17) (0.1 μM) binding were measured in the presence of 0.3–1.0 μM non-programmed ribosomes, and the rate constants, k_1 and k_{-1}, were calculated from the concentration dependence of the k_{app} of the reaction. The labile binding of the ternary complex to the ribosomes in the absence of mRNA or in the presence of a non-matching codon in the A-site reflects the initial codon-independent step of A-site binding that precedes codon recognition (Rodnina *et al*., 1996). The rate constants of the reaction, k_1 and k_{-1}, were shown to be independent of the mRNA (Pape *et al*., 1998). The calculations were performed by numerical intergration as described (Pape *et al*., 1998, 2000), and gave values of k_1 = 85 ± 15/s and k_{-1} = 30 ± 10/s.

The experiments of Figure 3B and C were carried out with 0.1 μM ternary complex and 0.3–1.0 μM 70S initiation complex. Rate constants were calculated from the combined sets of time courses for proflavin and mant-GTP fluorescence changes at four different ribosome concentrations. The data were fitted by numerical integration to a kinetic model depicted in Scheme 1 using Scientist for Windows (MicroMath Scientific Software) as described (Pape *et al*., 1998).

For the calculations, independently determined values of k_1 and k_{-1} were used (see above). The dissociation rate constant of the codon recognition complex, k_{-2}, is known to be small compared with the rate constant of the forward reactions (k_2 and k_3) (Eccleston *et al*., 1985; Pape *et al*., 1998). For the present purpose, the value of k_{-2} was estimated as 0.6/s by extrapolation of the previously obtained data (Pape *et al*., 1998) to the present ionic conditions. The rate of dipeptide formation, k_5, was determined separately from the data of Figure 6 (see below). The values of k_1, k_{-1}, k_{-2} and k_5 were fixed during the calculations of the remaining constants. The fitting yielded a unique solution for the rate constants k_2, k_3, k_4 and k_6. For values that were measured directly, the given standard deviation (Table I) was calculated from the results of several experiments. For values calculated by global fitting, the standard deviation for a given parameter was determined for the case when all other parameters, except the fixed ones, are allowed to change, i.e. if a given parameter was set to a value that is outside the range of the standard deviation, there was no fit satisfying all data sets.

The experiments of Figure 4 were performed by mixing 30S initiation complexes, 50S subunits and the ternary complex containing fluorescent labeled tRNA. Final concentrations of ternary complex, 50S subunits and the 30S initiation complex were 0.17, 0.6 and 0.3 μM, respectively. Time courses were fitted by numerical integration using Scientist for Windows. A model was used where Equation 2 was combined with the A-site binding model (scheme 1):

$$\text{30S complex} \xrightarrow{k_{GTP}} \text{70S complex} \xrightarrow{k_{Pi}} \text{B} \qquad (1)$$

which denotes GTP hydrolysis in IF2 upon joining of 50S subunits to the 30S initiation complex (k_{GTP}), followed by P_i release from IF2 after GTP hydrolysis (k_{Pi}) resulting in an elongation-competent state of the 70S initiation complex (B, see model 1).

To account for the delay in A-site binding in the experiments with GDP or in the absence of nucleotide in the initiation complex, the upper equation in model (2) was simplified to the following expression:

$$\text{30S complex} \xrightarrow{k_{transition}} \text{B} \qquad (2)$$

where $k_{transition}$ includes all the steps that take place after the joining of the 50S subunit and lead to the formation of the 70S initiation complex competent in A-site binding.

Dipeptide formation

The 30S initiation complexes were prepared as described above. Quench-flow experiments were performed in a KinTech RQF-3 apparatus. One sample syringe was loaded with pre-formed 30S initiation complex (0.3 μM) and EF-Tu·GTP·[^{14}C]Phe-$tRNA^{Phe}$ (0.6 μM) in the presence of either GTP or GDP (2 mM), and the other sample syringe was loaded with 50S subunits. Reactions were performed at 20°C and quenched with 0.2 M KOH. The quenched reaction mixtures were incubated for 15 min at 37°C, neutralized with acetic acid, centrifuged for 5 min at 12 000 r.p.m., and peptides were analyzed by HPLC. Since time courses of dipeptide formation were sigmoidal in the beginning, the data were analyzed by numerical integration using Scientist for Windows.

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