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Dissociation rates of peptidyl-tRNA from the P-site of *E.coli* ribosomes

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We studied the dissociation rates of peptidyl-tRNA from the P-site of poly(U)-programmed wild-type *Escherichia coli* ribosomes, hyperaccurate variants altered in S12 (SmD, SmP) and error-prone variants (Ram) altered in S4 or S5. The experiments were carried out in the presence and absence of streptomycin, and the effects of neomycin were tested in the wild-type ribosomes. Binding of peptidyl-tRNA to the P-site of wild-type ribosomes is much stronger than to their A-site. Addition of streptomycin dramatically reduces its affinity for the P-site. The S12 alterations make the P-site binding of peptidyl-tRNA much tighter, and the S4, S5 alterations make it weaker than in the case of the wild-type. We find that when binding of peptidyl-tRNA to the A-site is weak, then the affinity for the P-site is stronger, and vice versa. From these results, we formulate a hypothesis for the actions of streptomycin and neomycin based on deformations of the 16S rRNA tertiary structure. The results are also used to interpret some *in vivo* experiments on translational processivity.

Keywords: aminoglycoside/dissociation rate/peptidyl-tRNA/ribosome/rRNA

Introduction

There is a dichotomy in how ribosomes interact with aminoacyl-tRNA on the one hand and peptidyl-tRNA on the other. Aminoacyl-tRNA, both when in ternary complex with EF-Tu·GTP, and when alone, after dissociation of EF-Tu·GDP from the ribosome (Kaziro, 1978; Kurland *et al.*, 1995), requires a 'gentle' interaction with the programmed A-site (Kaziro, 1978) so that the accuracy of its selection is not obliterated by a strong non-codon-specific binding (Kurland and Ehrenberg, 1984, 1987; Kurland *et al.*, 1996). After peptidyl transfer the selection is over, and the peptidyl-tRNA must then be firmly bound in its A/P-state (Moazed and Noller, 1989; Noller, 1991) so that the growing polypeptide is not lost from the ribosome (Karimi and Ehrenberg, 1994; Kurland *et al.*, 1996). Binding of peptidyl-tRNA to the A-site (A/P-state) of wild-type ribosomes is quite strong (Karimi and Ehrenberg, 1994), and P-site (P/P-state according to Noller, 1991) binding of peptidyl-tRNA is normally even stronger (Erbe *et al.*, 1969; Leder *et al.*, 1969; Hamburger *et al.*, 1973). Antibiotics, like streptomycin (Sm) and neomycin (Nm), drastically increase missense errors (Davies and Davies, 1968; Ruusala and Kurland, 1984; Bilgin and Ehrenberg, 1994), and at the same time increase the binding strength of peptidyl-tRNA to the A-site (Karimi and Ehrenberg, 1994). Mutations in the *rpsL* gene, coding for protein S12, may lead to Sm dependence (SmD) (Gorini, 1971) as well as to pseudo-dependence (SmP) (Zengel *et al.*, 1977; Ruusala *et al.*, 1984). In the absence of Sm, SmD and SmP ribosomes are hyperaccurate *in vitro* (Bohman *et al.*, 1984; Ruusala *et al.*, 1984; Bilgin *et al.*, 1992; Bilgin and Ehrenberg, 1994), and the strength of peptidyl-tRNA binding to the A-site is weaker than for wild-type ribosomes (Karimi and Ehrenberg, 1994). The SmD phenotype may be reverted by mutations in the genes coding for ribosomal proteins S4 (*D12* and *D14*) (Gorini, 1971; Andersson and Kurland, 1983) and S5 (*rpsE1023*) (Piepersberg *et al.*, 1975; Andersson *et al.*, 1986). When these genes are expressed without the *rpsL* mutation, the ribosomes become error-prone (Gorini, 1971; Andersson and Kurland, 1983), and the A-site binding of peptidyl-tRNA is tighter than in the wild-type (Karimi and Ehrenberg, 1994).

The present work adds data to the findings on how the P-site binding of peptidyl-tRNA responds to the presence of the drugs Sm and Nm as well as to alterations in ribosomal proteins S12, S4 and S5. A striking, and unexpected, result is that there is an inverse relationship between the strength of peptidyl-tRNA binding to the A-site versus the P-site, such that strengthening the binding to one site by the action of Sm or Nm or by mutations weakens the affinity of peptidyl-tRNA for the other site.

We use these data to formulate a hypothesis to explain the mode of action of Sm and Nm. A key element of this hypothesis is that their activity induces conformational changes in 16S rRNA.

We also use the present findings to rationalize *in vivo* data on the processivity of ribosomes (Kurland *et al.*, 1996), under the influence of Sm (Menninger *et al.*, 1983; Caplan and Menninger, 1984), and with the Ram phenotype associated with alterations in ribosomal proteins S4 or S5 (Dong and Kurland, 1995).

Results

We have measured the rate of dissociaton of NAc[^{14}C]Phe-[^{3}H]Phe–tRNA^{Phe} (dip-tRNA) from the P-site of wild-type (017), hyperaccurate (SmD, SmP) and error-prone ribosomes (*D12*, *D14*, rpsE). We have also studied the effects of Sm on these rates and the effect of Nm on the dissociation of dip-tRNA from the P-site of 017 ribosomes.

Ribosomes were in most cases initiated with NAc[^{14}C]Phe-tRNA^{Phe} and poly(U) (Wagner *et al.*, 1982; Ehrenberg *et al.*, 1990). We also studied release of peptidyl-tRNA^{Phe} from the P-site (UUC codon) of wild-type ribosomes programmed with the mMFL mRNA ...AUGUUC-UUG... (Materials and methods). Incubation was started

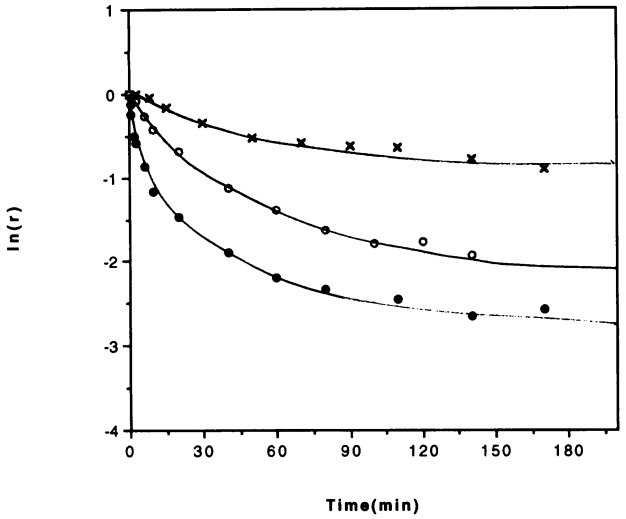

Fig. 1. The natural logarithm of the probability, r(t), that dip-tRNA remains in the P-site is plotted versus the time, t, after translocation. The experiment was carried out at 37°C and shows ln(r) for wt (○), D14 (●) and SmD (×).

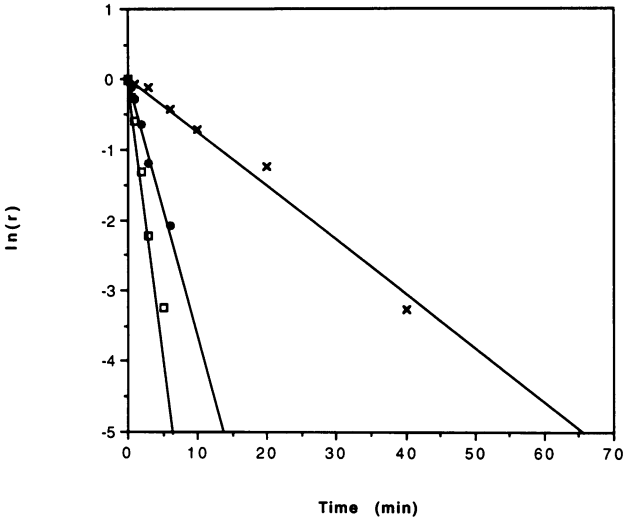

Fig. 2. The effects of Sm and Nm on the dissociation of dip-tRNA from the P-site at 37°C. ln(r) is plotted versus the time, t, after translocation as in Figure 1 for wt ribosomes with no drug (×), wt + Sm (●) and wt + Nm (□).

by adding translation factors, including elongation factor G (EF-G), [^{3}H]Phe-tRNA^{Phe} and non-acylated tRNA^{Phe} to the pre-initiated ribosomes. The amount of active ribosomes (12.5 pmol) was kept in excess over the available amount of [^{3}H]Phe-tRNA^{Phe} (3 pmol), by adding a limiting amount of [^{3}H]Phe (3 pmol) to the factor mix (Materials and methods). After peptidyl transfer, dip-tRNA rapidly moves to the P-site by the action of EF-G. HPLC analysis demonstrated the existence of NAc[^{14}C]Phe–[^{3}H]Phe and showed that the amounts of tri- or tetrapeptides can be neglected compared with the amount of dipeptides (not shown). After different incubation times (t), a large excess of unlabelled ternary complex, containing Phe-tRNA^{Phe}, was added to start synthesis of long poly(Phe) chains. Translation was stopped after 1 min by the addition of trichloroacetic acid (TCA). Those NAc[^{14}C]Phe–[^{3}H]Phe-tRNA^{Phe} molecules that remained in the P-site at time 't' were detected subsequently as hot TCA-precipitable ^{3}H counts (Materials and methods).

Typical experiments are shown in Figure 1 for wild-type ribosomes as well as for the error-prone variant *D14* (Ram, altered in S4) and the hyperaccurate variant SmD (altered in S12). The plots show the natural logarithm, ln(r), of the amount of hot TCA-precipitable [^{3}H]Phe at incubation time t, divided by the corresponding amount at time zero. This ratio, r(t), can be interpreted as the probability that dip-tRNA remains in the P-site at time t after translocation. The curves reveal that the dissociation rate of dip-tRNA from Ram ribosomes is faster than from wild-type, and is faster from wild-type than from SmD ribosomes. Interestingly, dissociation of dip-tRNA from the A-site of these variants shows the opposite pattern (Karimi and Ehrenberg, 1994). Dip-tRNA dissociates more quickly from the A-site of SmD than from the A-site of wild-type ribosomes, and more quickly from wild-type than from *D14*. In these cases the binding strength of dip-tRNA to the A-site is negatively correlated with the affinity to the P-site: when the binding of dip-tRNA to the A-site is strengthened (by a mutation), the binding to the P-site is reduced.

Figure 1 also shows that the dissociation of dip-tRNA from the P-site of the different ribosome variants cannot be described as a single exponential, in which case the plot would have been a straight line. In contrast, dissociation of dip-tRNA from the A-site is truly first order and occurs as a single exponential (Karimi and Ehrenberg, 1994). Dissociation of fMet–Phe-tRNA^{Phe} from wild-type ribosomes with the UUC codon in the P-site (mMFL RNA) is somewhat faster than from the P-site of poly(U)-programmed ribosomes. Also here two dissociation rates are visible (Table I).

The experiments in Figure 1 are summarized in Table I, along with results from equivalent experiments performed on another hyperaccurate variant (SmP, altered in S12) and from the Ram ribosomes *D12* (altered in S4) and *rpsE* (altered in S5). The dissociation curves could be fitted by one (for SmP) or by two (for all other cases) exponential(s). Fast and slow rate constants and their correponding amplitudes are given in Table I, where we also show the average times, t_{diss}, that dip-tRNA remains in the P-site in the different cases. When the dissociation is a single exponential, then t_{diss} is simply the reciprocal of the dissociation rate constant. In general, t_{diss} is calculated by integrating the ratio r(t) from zero to infinite time (Bilgin *et al.*, 1992). For comparison, t_{diss} values for A-site binding (Karimi and Ehrenberg, 1994) are also included in the table.

For wild-type ribosomes, the table shows that peptidyl-tRNA is more stably bound to the P- ($t_{diss} = 1.38\times10^3$ s) than to the A-site ($t_{diss} = 0.11\times10^3$ s), in accordance with the literature (Erbe *et al.*, 1969; Leder *et al.*, 1969; Hamburger *et al.*, 1973). This tendency is enhanced for the SmP and SmD ribosomes, where the t_{diss} values for P-site binding of peptidyl-tRNA are ~200-fold longer than for their A-site counterparts: for these hyperaccurate ribosomes, P-site binding is stabilized in relation to the wild-type, and A-site binding is destabilized.

In contrast, for the three Ram variants, P-site binding is destabilized in relation to the wild-type so that for *D14*

Table I. Average time (t_{diss}) and dissociation rates (k_d) of dip-tRNA for wild-type and mutant ribosomes

Ribosome	P-site					A-site[a]
	Average time ($t_{diss} \times 10^{-3}$ s)	Fast dissoc. rate constant $k_d \times 10^3$/s	Fast amplitude (%)	Slow dissoc. rate constant $k_d \times 10^3$/s	Slow amplitude (%)	Average time $t_{diss} \times 10^{-3}$ s
wt	1.38	1.8	52	0.43	48	0.11
wt[b]	0.42	3.3	34	2.1	66	–
SmD	15.6	0.7	40	0.04	60	0.079
SmP	19.8	–	0	0.05	100	0.091
rpsE	0.96	14	48	0.55	52	–
D14	0.66	10	61	0.68	39	0.50
D12	0.42	21	37	1.60	63	–

[a]From Karimi and Ehrenberg (1994).
[b]Experiment using the heteropolymer mRNA (...AUGUUCUUG...).

Table II. Average time (t_{diss}) and dissociation rates (k_d) of dip-tRNA for wild-type and mutant ribosomes in the presence of streptomycin

Ribosome	P-site					A-site[a]
	Average time ($t_{diss} \times 10^{-3}$ s)	Fast dissoc. rate constant $k_d \times 10^3$/s	Fast amplitude (%)	Slow dissoc. rate constant $k_d \times 10^3$/s	Slow amplitude (%)	Average time $t_{diss} \times 10^{-3}$ s
wt	0.17	6.0	100	–	0	0.36
wt[b]	0.084	12	100	–	0	0.71
SmD	0.78	11	49	0.70	51	0.38
SmP	0.96	7.4	51	0.56	49	0.42
rpsE	0.84	1.2	100	–	0	–
D14	0.42	2.3	100	–	0	0.83
D12	0.36	2.7	100	–	0	–

[a]Values from Karimi and Ehrenberg (1994).
[b]Experiment adding neomycin instead of streptomycin.

ribosomes the dissociation time of dip-tRNA from the P-site ($t_{diss} = 0.66 \times 10^3$ s) is similar to the time of dissociation from the A-site ($t_{diss} = 0.50 \times 10^3$ s). For these Ram ribosomes, therefore, the affinities of peptidyl-tRNA for the A- and P-sites appear to be 'balanced'.

Addition of Sm or Nm dramatically enhances the dissociation rate of dip-tRNA from the P-site (Figure 2, Table II). For wild-type ribosomes, t_{diss} decreases from 1.38×10^3 s in the absence of any drug to 0.17×10^3 s in the presence of Sm and to 0.084×10^3 s in the presence of Nm (Table II). Remarkably, in these two cases, A-site is much stronger than P-site binding (Table II). This finding adds another phenotypic effect to the already long list of actions that characterize these two important antibiotics.

Dissociation of dip-tRNA from the P-site in the presence of Sm appears in most cases to be a simple first order process, which can be described by a single exponential. The exceptions are SmD and SmP ribosomes, where two exponentials are necessary to reproduce experimental data (Table II).

Sm significantly reduces the affinity of P-site binding (as measured from the t_{diss} value) in all cases except for the three Ram ribosomes (Tables I and II). Here the effect of the drug is subtle and quite interesting. In the absence of Sm, P-site dissociation occurs with two rates for these variants. The fast rate is $\sim 15 \times 10^{-3}$/s and the slow one $\sim 1 \times 10^{-3}$/s (Table I). In the presence of Sm, in contrast, there is a single rate which is $\sim 2 \times 10^{-3}$/s (Table II).

Discussion

Binding of the antibiotic Sm to *E.coli* ribosomes changes their interaction with tRNA throughout the various steps of protein elongation, and these changes have dramatic repercussions on ribosome function. Sm reduces the accuracy of aminoacyl-tRNA selection by orders of magnitude (Gorini, 1965; Ruusala and Kurland, 1984), first, by interfering with the ternary complex–ribosome interaction and thereby reducing the 'initial selection' of these tRNAs (Ruusala *et al.*, 1982; Bilgin *et al.*, 1992; Bilgin and Ehrenberg, 1994). Secondly, Sm interferes with the 'proof-reading' (Hopfield, 1974; Ninio, 1975) of aminoacyl-tRNA (Ruusala *et al.*, 1982; Thompson and Karim, 1982) in a step following GTP hydrolysis on EF-Tu but preceding peptidyl transfer (Ruusala and Kurland, 1984; Bilgin *et al.*, 1992; Bilgin and Ehrenberg, 1994). Recent experiments indicate that the error enhancement by Sm is due partially to a general stabilization of aminoacyl-tRNAs in the A-site (Karimi and Ehrenberg, 1994), but that the main effect stems from a severe distortion of codon–anticodon interactions, making the standard free energy of the RNA–RNA duplex quite insensitive to base mismatches (Ehrenberg *et al.*, 1996; R.Karimi and M.Ehrenberg, in preparation) as previously suggested by Cundliffe (1981). Further down the line in the elongation cycle, Sm significantly reduces the rate at which peptidyl-tRNA is translocated from the A- to the P-site (Bilgin *et al.*, 1992). To these events can now be added that Sm dramatically

destabilizes the binding of peptidyl-tRNA to the P-site (Tables I and II and Figure 2).

Why Sm has such a rich action spectrum may be guessed from chemical protection data and by comparing the effects of Sm with those of another antibiotic, Nm. Sm induces chemical protection of bases 913–915 of 16S rRNA (Moazed and Noller, 1987), indicating an RNA-mediated binding of the drug in this region. Nm, in contrast, confers protection of bases 1408–1409, and binding occurs to a segment of 16S rRNA including these bases (Moazed and Noller, 1987). Despite having putatively different binding sites, Sm and Nm have similar effects on missense errors (R.Karimi, unpublished), translocation (Cundliffe, 1981; Bilgin *et al.*, 1992), and on A-site (Karimi and Ehrenberg, 1994) and P-site binding of peptidyl-tRNA (Table II and Figure 2). This indicates that the action of both drugs is indirect and is caused by a similar type of distortion of 16S rRNA, albeit induced from two different places in the primary sequence.

Moazed and Noller (1987) identified a set, III, of several sites in 16S rRNA, that is protected from dimethyl sulfate modification by tRNA as well as by 50S binding to the 30S ribosomal subunit. Interestingly, Nm binding leads to the same protection pattern as for tRNA and 50S, while binding of Sm only protects a subset of class III sites. This indicates that Sm and Nm may be similar in some respects, for example in how they influence peptidyl-tRNA binding to A- and P-site, but different in others. In line with this, preliminary experiments *in vivo* with ribosome mutants reveal large, functional differences in the action of the two drugs (R.Karimi, in preparation).

There are several types of Sm resistance, and of particular interest in the present context are those that lead to Sm dependence (SmD) (Gorini, 1971) or Sm pseudo-dependence (SmP) (Zengel *et al.*, 1977; Andersson and Kurland, 1983), through alterations in ribosomal protein S12. These ribosomes have similar *in vitro* phenotypes. They are hyperaccurate (Ruusala *et al.*, 1984; Bilgin *et al.*, 1992; Bilgin and Ehrenberg, 1994) and they have weaker A-site (Karimi and Ehrenberg, 1994) and much stronger P-site binding (Table I and Figure 1) of peptidyl-tRNA than wild-type. In other words, their phenotype is a 'complementary image' of the phenotype induced by Sm. When Sm is added to these ribosomes they become similar to wild-type with respect to all these properties (Ruusala *et al.*, 1984; Bilgin *et al.*, 1992; Bilgin and Ehrenberg, 1994; Karimi and Ehrenberg, 1994; Table II). From this type of data we suggest that amino acid substitutions in S12, that lead to the SmP and SmD phenotypes, distort 16S rRNA in the 'opposite direction' from the Sm-induced perturbation. When the drug is present, the two 'opposing pulls' are balanced, with wild-type-like structure and function as a result.

An amino acid substitution in S12, leading to the SmD phenotype, can be complemented by alterations in S4 (Gorini, 1971; Andersson and Kurland, 1983) or S5 (Piepersberg *et al.*, 1975; Andersson *et al.*, 1986). Expression of the latter mutations, without the S12 change, give error-prone ribosomes (Gorini, 1971; Andersson and Kurland, 1983), stable A-site (Karimi and Ehrenberg, 1994) and unstable P-site (Table I and Figure 1) binding of peptidyl-tRNA. In other words, the S4 and S5 changes that compensate for Sm dependence, make the ribosomes behave very much like wild-type in complex with Sm. We suggest, therefore, that these S4 and S5 changes distort 16S rRNA in the 'same direction' as Sm (or Nm). One, testable, prediction from this hypothesis is that these Ram ribosomes have impaired EF-G function, like wild-type ribosomes in complex with Sm (Bilgin *et al.*, 1992) or Nm.

The ultimate test of the present hypotheses, concerning the action of Sm and Nm as well as of pertinent amino acid substitiutions in S4, S5 and S12, has to await high resolution models of the 16S rRNA tertiary structure (Brimacombe, 1991; Noller, 1991).

The present results have consequences not only for the drug-enforced evolution of the 16S rRNA tertiary domains, but also suggest new interpretations of *in vivo* experiments.

According to Menninger *et al.* (1983) and Caplan and Menninger (1984), Sm not only raises missense error levels (Ruusala and Kurland, 1984), but it also causes increased drop-off of peptidyl-tRNA from the ribosome. We suggest that this enhanced drop-off rate may be explained by the much weaker P-site binding of peptidyl-tRNA in the presence of Sm (Tables I and II and Figure 2).

Dong and Kurland (1995) found that Ram ribosomes have lower processivity than wild-type, and when they ranked the strains according to decreasing processivity they found the following order: wild-type, *rpsE*, *D14* and *D12*. This ranking order does not agree with how the level of missense errors varies with ribosomal phenotype. Here *D14* has the highest error rate, followed by *rpsE*, *D12* and finally wild-type (Andersson *et al.*, 1986; R.Karimi, unpublished). In contrast, when these ribosomes are ordered with respect to the average time, t_{diss}, that their peptidyl-tRNAs remain in the P-site, a ranking identical to that for the decreasing processivity is obtained (Table I). This is suggestive, and may mean that the observations by Dong and Kurland (1995) concerning Ram ribosomes can be rationalized as drop-off events from the P-site. In contrast, the reduced processivity of hyperaccurate ribosomes (Jörgensen and Kurland, 1990; Dong and Kurland, 1995), altered in S12, probably reflects drop-off from the ribosomal A-site (Karimi and Ehrenberg, 1994; Kurland *et al.*, 1996).

Finally, we must adress a complication in our dataset (Figures 1 and 2, Tables I and II). In many cases, the dissociation of peptidyl-tRNA from the P-site does not follow first order kinetics, but must be described using two exponentials (Tables I and II). This may have the trivial explanation that our ribosomes are intrinsically heterogeneous, but the data suggest a more interesting interpretation. It is thus conceivable that the peptidyl-tRNA, immediately after translocation, enters a first state of the P-site. From this, peptidyl-tRNA may either dissociate with a fast rate or move to a more stable P-state, from which dissociation is slower. It is possible, furthermore, that the second ternary complex, involved in translation (Ehrenberg *et al.*, 1990; Weijland and Parmeggiani, 1993; Weijland *et al.*, 1994; Dincbas *et al.*, 1995) catalyses the transition between the two different P-states.

Materials and methods

Chemicals

Poly(U), GTP and ATP were purchased from Pharmacia (Uppsala). Phospho*enol*pyruvate, putrescine, spermidine, myokinase, L-phenyl-

alanine, Sm sulfate salt and Nm sulfate salt were from Sigma. Pyruvate kinase was from Boehringer (Mannheim). [^{3}H]phenylalanine, [^{14}C]-phenylalanine and [^{3}H]methionine were from Amersham. All other chemicals of analytical grade were from Merck.

Buffers

Polymix (Jelenc and Kurland, 1979) was composed of 5 mM magnesium acetate, 0.5 mM $CaCl_2$, 5 mM NH_4Cl, 95 mM KCl, 8 mM putrescine (pH 7.5), 1 mM spermidine, 5 mM potassium phosphate (pH 7.3) and 1 mM dithioerythritol (DTE). A 10-fold concentrated polymix was prepared and stored without potassium phosphate and DTE to avoid precipitation, and the correct working strength was obtained by adding from stock solutions of 100 mM potassium phosphate and 50 mM DTE in the experiments.

Buffer A for HPLC contained 10% methanol, 0.1% trifluoroacetic acid and Buffer B contained 90% methanol, 0.1% trifluoroacetic acid.

Strains and biochemicals

Escherichia coli strains 017 (wild-type) and S12 mutants, derived from 017, UK318 (SmP) and UK666 (SmD), have been described by Ruusala *et al.* (1984). The S4 mutants derived from *D12* and *D14* (Ram) were described by Olsson and Isaksson (1979). The S5 mutant derived from *rpsE1023* (ram) was described by Piepersberg *et al.* (1975). The ribosomes were prepared (Jelenc, 1980) and kept in polymix buffer at −80°C. Purification of enzymes and other components used in the *in vitro* poly(U) system have been described by Ehrenberg *et al.* (1990). Preparation of the 83 nucleotide long mMFL mRNA with coding sequence AUGUUCUUG... (Met–Phe–Leu) and with the ribosome binding site from the 002 mRNA sequence (Calogero *et al.*, 1988) was prepared by *in vitro* transcription of an artificial gene (V.Dincbas, M.Pavlov, N.Bilgin and M.Ehrenberg, manuscript in preparation). Initiation factors were purified from overproducing strains according to Soffientini *et al.* (1994). [^{3}H]fMet-tRNA was prepared following the protocol of Hershey and Trash (1967) with minor modifications.

Measurement of the dissociation rate of dip-tRNA from the P-site of poly(U)-programmed ribosomes

Three mixes were prepared on ice. First, a 70S mix was prepared containing, in 50 µl of polymix, 12.5 pmol of active ribosomes, Ac[^{14}C]Phe-tRNA^{Phe} (sp. act. 1 c.p.m./pmol) 20% in excess over the total amount of ribosomes (~60 pmol) according to Wagner *et al.* (1982), 25 µg of poly(U) and 100 nmol of GTP. Where indicated, Sm (10 µg) or Nm (0.1 µg) were added. The fraction of active ribosomes is defined as the number of elongating ribosomes (measured from Ac[^{3}H]Phe precipitable by hot TCA) divided by the total number of ribosomes (measured by absorbance).

Second, a labelled factor mix was prepred containing, in 40 µl of polymix, 80 nmol of ATP, 0.8 µmol of phospho*enol*pyruvate, 5 µg of pyruvate kinase, 0.3 µg of myokinase, 3.3 pmol of [^{3}H]phenylalanine (400 c.p.m./pmol), 20 units of Phe-tRNA synthetase (one unit of synthetase aminoacylates 1 pmol of tRNA^{Phe} per s), 1500 pmol of tRNA^{Phe}, 25 pmol of EF-Tu, 50 pmol of EF-Ts and 5 pmol of EF-G.

Third, an unlabelled factor mix was prepared containing, in 10 µl of polymix, 20 nmol of ATP, 0.2 µmol of phospho*enol*pyruvate, 30 nmol of phenylalanine, 200 pmol of tRNA^{Phe}, 300 pmol of EF-Tu, 100 units of Phe-tRNA synthetase and 50 pmol of EF-Ts.

The mixes were incubated for 10 min before 40 µl of factor mix were added to 50 µl of 70S mix for different incubation times (Results). After incubation, 10 µl of unlabelled factor mix were added, and poly(Phe) synthesis was stopped after 1 min in all cases with 5 ml of 5% TCA containing 15% casamino acids (Difco). The samples were incubated for 10 min at 95°C and filtered through glass filters (GF/C). These were washed with 5% TCA, then with propanol, dried and the radioactivity measured (Ehrenberg *et al.*, 1990).

Measurement of the dissociation rate of dip-tRNA from the P-site of heteropolymer-programmed ribosomes

Three mixes were prepared on ice. First, a 70S mix was prepared containing, in 50 µl of polymix, 12.5 pmol of active ribosomes, 100 pmol of mMFL mRNA (...AUGUUCUUG...), 100 pmol of f[^{3}H]Met-tRNA^{fMet} (sp. act. 400 c.p.m./pmol), 50 pmol of initiation factor 1, 10 pmol of initiation factor 2, 50 pmol of initiation factor 3 and 100 nmol of GTP.

Second, a factor mix was prepared containing, in 40 µl of polymix, 80 nmol of ATP, 0.8 µmol of phospho*enol*pyruvate, 5 µg of pyruvate kinase, 0.3 µg of myokinase, 30 pmol of [^{14}C]phenylalanine (5 c.p.m./pmol), 20 units of Phe-tRNA synthetase, 1500 pmol of tRNA^{Phe}, 60 pmol of EF-Tu, 50 pmol of EF-Ts and 5 pmol of EF-G.

Third, an unlabelled factor mix was prepared containing, in 10 µl of polymix, 20 nmol of ATP, 0.2 µmol of phospho*enol*pyruvate, 3 nmol of leucine, 100 pmol of tRNA$^{Leu}_4$, 100 pmol of EF-Tu, 20 units of Leu-tRNA synthetase and 50 pmol of EF-Ts.

The mixes were incubated for 10 min before 40 µl of factor mix were added to 50 µl of 70S mix for different incubation times. After incubation, 10 µl of the third factor mix were added, and tripeptide formation was stopped after 1 min with 20% formic acid. The precipitate was pelleted by Eppendorf centrifugation and 220 µl of 0.5 M KOH were added to each pellet. The fMet–Phe–Leu-tRNA was hydrolysed to tripeptide and deacylated tRNA for 45 min at 37°C. Subsequently, 10 µl of concentrated HCOOH were added and precipitable material was separated from tripeptide by Eppendrof centrifugation. Then 160 µl of supernatant were analysed for tripeptide content by HPLC, as described by Ehrenberg *et al.* (1990).

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