Conformational switch in the decoding region of 16S rRNA during aminoacyl-tRNA selection on the ribosome

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Binding of aminoglycoside antibiotics to 16S ribosomal RNA induces a particular structure of the decoding center and increases the misincorporation of near-cognate amino acids. By kinetic analysis we show that this is due to stabilization of the near-cognate codon recognition complex and the acceleration of two rearrangements that limit the rate of amino acid incorporation. The same rearrangement steps are accelerated in the cognate coding situation. We suggest that cognate codon recognition, or near-cognate codon recognition augmented by aminoglycoside binding, promote the transition of 16S rRNA from a 'binding' to a 'productive' conformation that determines the fidelity of decoding.

The ribosome is composed of two subunits — the small subunit (30S in bacteria) that is the site of codon–anticodon interaction and the large subunit (50S) that catalyzes peptide bond formation between aminoacyl-transfer RNA (aa-tRNA) bound to the A site and peptidyl-tRNA bound to the P site. During translation, the aa-tRNA is bound to the A site in a GTP-dependent process that is catalyzed by elongation factor Tu (EF-Tu). Aminoacyl-tRNA (aa-tRNA) selection in response to the codon in the ribosomal A site takes place at two points: before GTP hydrolysis of EF-Tu (initial selection) and after GTP hydrolysis but before peptide bond formation (proofreading). During initial selection, noncognate aa-tRNA•EF-Tu•GTP complexes that do not induce GTP hydrolysis are rejected. When a cognate codon is recognized, that is, when three base pairs are formed between codon and anticodon, the GTPase of EF-Tu is activated, GTP is hydrolyzed and aa-tRNA is released from EF-Tu•GDP[1]. The aminoacyl end of aa-tRNA is then free to move into the peptidyl transferase center on the 50S subunit (accommodation) and to take part in peptide bond formation (Fig. 1). A similar sequence of events occurs when a near-cognate codon is recognized, that is, when the codon–anticodon match is imperfect. However, in such a case, following GTP hydrolysis and release from EF-Tu, near-cognate aa-tRNA dissociates from the ribosome rather than entering the peptidyl transferase center. The efficiency of near-cognate aa-tRNA rejection in initial selection and proofreading is determined by both the lower stability of the codon–anticodon complex and slower forward reactions (GTPase activation and accommodation), compared to the cognate situation[2].

The decoding center of 16S ribosomal RNA (rRNA) has an essential role in maintaining the fidelity of aa-tRNA selection by the ribosome[3–5]. Aminoglycoside antibiotics such as paromomycin, gentamicin and neomycin decrease the fidelity of decoding[6] by binding to the A site of the decoding center[7] and inducing a structural change of the binding site[7,8]. These findings indicate a link between the conformation of 16S rRNA and decoding. The antibiotics are bound in the major groove of the A site rRNA within a pocket created by the A1408•A1493 base pair and the bulged A1492. The three adenines are protected from dimethyl sulfate (DMS) modification by mRNA-dependent tRNA binding to the A site, indicating that the decoding center is probably in direct contact with the codon–anticodon complex[9–12]. Therefore, it has been suggested that paromomycin induces a conformation of 16S rRNA that binds tRNA to the A site with high affinity[7]. This would increase the error frequency by stabilizing the binding of aa-tRNA[13]. Indeed, it has been reported that the antibiotic decreases the rate of tRNA dissociation from the A site about six-fold[14]. However, this effect is too small to explain the aminoglycoside-induced increase of misreading, which ranges from 10- to 200-fold, depending on the codon and the antibiotic[6]. Thus, it appears that additional effects are involved. This is addressed here by kinetic analysis.

To first assess the effect of paromomycin on the initial stage of discrimination of noncognate ternary complexes, GTP hydrolysis rates were measured when the ternary complex, EF-Tu•GTP•Phe-tRNA^{Phe}, was bound to ribosomes programmed with different mRNAs. With poly(A)-programmed ribosomes that display a noncognate AAA codon in the A site, the antibiotic enhanced the rate of GTP hydrolysis about two-fold, with a concomitant increase of K_M by about a factor of two (Fig. 2). The same result was obtained with unprogrammed, vacant ribosomes. Similarly, GTP hydrolysis in the binary complex, EF-Tu•GTP, on the ribosome was stimulated about two-fold (data not shown). Hence, paromomycin increases the k_{cat}/K_M value for noncognate ternary complexes about four times, because of the two-fold increase of both k_{cat} and K_M of the GTPase reaction. There is no significant change of k_{cat}/K_M in the cognate coding situation with poly(U) (88 *versus* 86 μM^{-1} s^{-1}), as calculated from elemental rate constants determined in the absence and presence of paromomycin (ref. 2 and data not shown). Initial selectivity is defined as $(k_{cat}/K_M)_{correct}/(k_{cat}/K_M)_{incorrect}$ of the GTPase; hence paromomycin in the noncognate poly(A) system

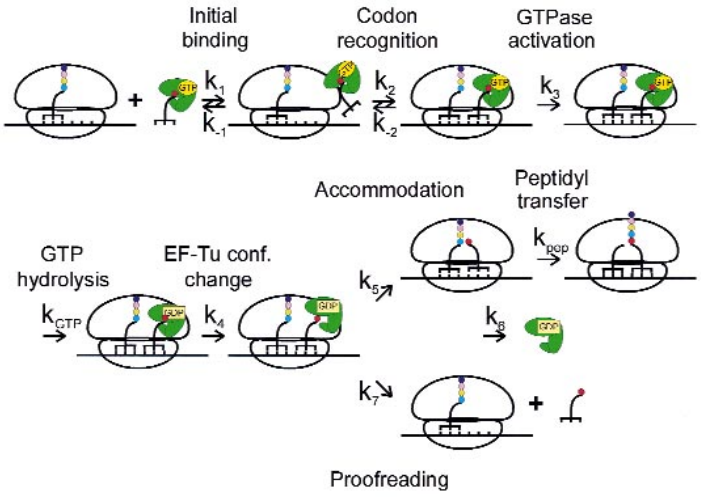

Fig. 1. Kinetic scheme of aa-tRNA binding to the ribosomal A site. EF-Tu (light green) is depicted in three conformations: the GTP form with aa-tRNA bound to it, the transient GTPase-activated form on the ribosome (G domain dark green) and the GDP-bound form that dissociates from the ribosome.

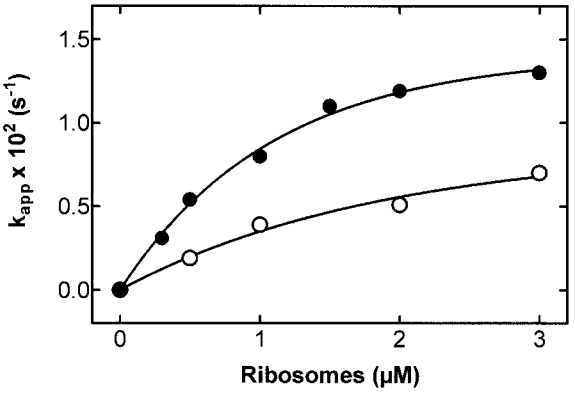

Fig. 2 Paromomycin effect on GTP hydrolysis. Values for k_{app} of GTP hydrolysis in the ternary complex, EF-Tu•GTP•Phe-tRNA^{Phe}, on poly(A)-programmed ribosomes were measured in the absence (●) and presence (●) of paromomycin. Ternary complex (0.1 μM) and ribosomes were incubated at 20°C for up to 30 min; apparent rate constants were obtained by single-exponential fitting. Saturation levels represent first-order rate constants of GTP hydrolysis, 0.008 s^{-1} and 0.014 s^{-1}, in the absence and presence of paromomycin, respectively.

reduces the initial selectivity about four-fold.

In order to study the influence of paromomycin on GTP hydrolysis in different coding situations, we used mRNA-programmed 70S initiation complexes displaying various codons in the A site. To these complexes, ternary complex (EF-Tu•GTP•Phe-tRNA^{Phe}) was added, and the rate of GTP hydrolysis was measured in the absence and presence of paromomycin. In the absence of antibiotic, GTP hydrolysis was slow (0.002–0.003 s^{-1}) with all noncognate codons (no base pair possible) as well as with the UUG:AAG pair featuring a guanine-guanine apposition in the third codon position (Leu 5; Table 1). In these cases, where anticodon and codon do not match sufficiently to induce fast GTPase, paromomycin slightly (up to three-fold) stimulated EF-Tu-dependent GTP hydrolysis on the ribosome (Table 1). Similarly, for the near-cognate CUU:AAG complex, with a mismatch in the first and a wobble base pair in the third codon position, the stimulation by paromomycin of the rate of GTP hydrolysis was about four-fold (Table 1). These effects are small in view of the reported finding that paromomycin strongly increases the misreading of near-cognate codons[15].

In order to quantitatively assess the effect of paromomycin on ribosomal accuracy, we have analyzed in full the kinetics of A site binding of near-cognate ternary complex in the presence

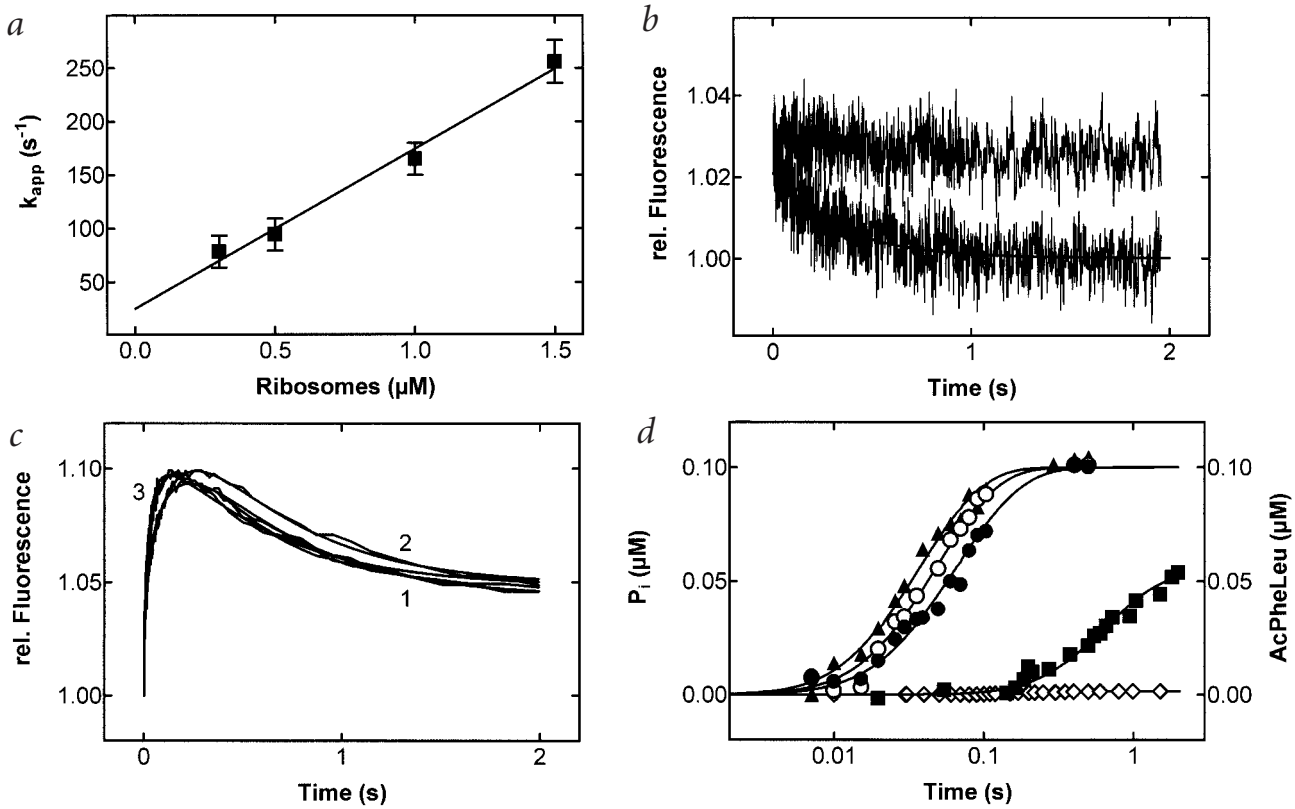

Fig. 3. Kinetics of Leu 2 binding in the presence of paromomycin. ***a***, Initial binding. k_{app} values were obtained by exponential fitting of time courses of EF-Tu•GTP•Leu-tRNA^{Leu2}(Prf16/17/20) binding to poly(A)-programmed ribosomes measured by fluorescence stopped flow. The dependence of k_{app} on ribosome concentration gives $k_1 = 140 \pm 20$ μM^{-1} s^{-1} (slope) and $k_{-1} = 25 \pm 10$ s^{-1} (ordinate intercept). ***b***, Determination of k_{-2}. The codon recognition complex was prepared from EF-Tu•GDPNP•Leu-tRNA^{Leu2}(Prf16/17/20) and poly(U)-programmed ribosomes with AcPhe-tRNA^{Phe} in the P site. The dissociation of the complex was initiated by adding of a 10-fold excess of nonfluorescent EF-Tu•GDPNP•Phe-tRNA^{Phe} and monitored in the stopped-flow apparatus. Single-exponential fitting yields $k_{-2} = 3.5 \pm 0.5$ s^{-1}. ***c***, Conformational changes of aa-tRNA. A-site binding was monitored by the fluorescence of Leu-tRNA^{Leu2}(Prf16/17/20). Time courses obtained with 0.3 (1), 0.5 (2) and 1.5 μM (3) of poly(U)-programmed, P site-blocked ribosomes are shown. ***d***, GTP hydrolysis and dipeptide formation. GTP hydrolysis by EF-Tu•[γ-^{32}P]GTP•[^{3}H]Leu-tRNA^{Leu2} was measured with 0.3 μM (●), 0.5 μM (○)and 1.5 μM (▲) poly(U)-programmed, P site-blocked ribosomes; AcPhe-[^{3}H]Leu formation with 1.5 μM ribosomes (■); dipeptide formed in the absence of antibiotic is shown for comparison (◇)[2]. Data sets used for global fitting were combined from data shown in (*c*) and (*d*) as well as from additional data obtained at 1.0 μM and 2.0 μM ribosomes. Smooth lines in *c* and *d* are calculated from elemental rate constants (Table 2).

Table 1 Effect of paromomycin on GTP hydrolysis

Codon 5' to 3' (tRNA)	GTP hydrolysis rate[1] (s^{-1})	
	-Paromomycin	+Paromomycin
AGA (Arg)	0.002 ± 0.001	0.003 ± 0.001
UCA (Ser)	0.003 ± 0.001	0.007 ± 0.001
UAU (Tyr)	0.003 ± 0.001	0.003 ± 0.001
UUG (Leu5)	0.002 ± 0.001	0.007 ± 0.001
CUC (Leu2)	0.07 ± 0.02	0.29 ± 0.01
UUU (Phe)	6.5 ± 0.1	3.8 ± 0.3

[1]EF-Tu•[γ-^{32}P]GTP•Phe-tRNA^{Phe} (anticodon 3'-AAG-5') (0.1 μM) was bound to mRNA-programmed 70S initiation complexes (0.2 μM; for CUC and UUU, 1 μM) displaying various codons in the A site and GTP hydrolysis measured as described in Methods.

of paromomycin. For the kinetic analysis, we used poly(U)-programmed ribosomes and Leu-tRNA^{Leu2} (anticodon GAG), because a complete set of rate constants was available for this system[2]. Kinetic data were obtained by fluorescence stopped flow using proflavin-labeled Leu-tRNA^{Leu2}(Prf16/17/20) and by quench flow (GTP hydrolysis and dipeptide formation). In parallel, the kinetic effects of paromomycin in the cognate situation with Phe-tRNA^{Phe} were also analyzed. In order to allow the evaluation of the kinetic scheme (Fig. 1) by fitting (see below), three rate constants (k_1, k_{-1} and k_{-2}) were measured directly and used as fixed values in the fit. Those rate constants were determined as follows.

Codon-independent initial binding of EF-Tu•GTP•Leu-tRNA^{Leu2} to ribosomes, characterized by rate constants k_1 and k_{-1} (ref. 16), was measured at different concentrations of ribosomes programmed with poly(A). Apparent rate constants (k_{app}) were obtained by exponential fitting, and rate constants were determined from the linear concentration dependence of k_{app} (Fig. 3*a*). For the association rate constant, k_1, a value of 140 μM^{-1} s^{-1} was obtained in the presence of paromomycin that was comparable to the value obtained in the absence of antibiotic, 110 μM^{-1} s^{-1} (ref. 2). The dissociation rate constant, k_{-1}, was the same with or without paromomycin (25 s^{-1}).

The dissociation rate constant of the codon recognition complex, k_{-2}, was determined by measuring the dissociation rate of the complex formed in the presence of a non-hydrolyzable GTP analog, GDPNP. In that complex, A site binding is blocked after codon recognition, while the kinetics of initial binding and codon recognition are unaffected[17]. The preformed codon recognition complex, EF-Tu•GDPNP•Leu-tRNA^{Leu2} (Prf16/17/20) on poly(U)-programmed ribosomes was rapidly mixed with a 10-fold excess of unlabeled EF-Tu•GDPNP•Phe-tRNA^{Phe} in the stopped-flow apparatus. A single-exponential fluorescence decrease to the level of unbound ternary complex was observed (Fig. 3*b*). The value of the time constant obtained (k_{-2} = 3.5 s^{-1}) is about five times smaller than the value obtained in the absence of the antibiotic (17 s^{-1}; ref. 2), indicating a stabilization by paromomycin of the codon recognition complex.

The remaining elemental rate constants of the scheme depicted in Fig. 1 were obtained by global fitting (refs 1,2); that is, all time courses measured with different observables at various ribosome concentrations (Fig. 3*c*,*d*) were included in the fit. The rate constants k_4, k_5 and k_7 were also obtained analytically from the length of the lag phase, the rate, and the extent of dipeptide formation (Fig. 3*d*). Rate constants are summarized in Table 2. For comparison, the previously determined rate constants of Leu-tRNA^{Leu2} binding to the A site in the absence of antibiotic[2], which were confirmed here, are included. Note that paromomycin affects all steps, except initial binding (step 1). In agreement with previous data[14], the stability of the codon–anticodon interaction is increased as evident from the five- to six-fold decrease of the rate constants of both ternary complex (k_{-2}) and aa-tRNA (k_7) dissociation. Furthermore, k_2, the rate constant of codon recognition, is reduced about three times. The same effect of paromomycin was observed with cognate ternary complex containing Phe-tRNA^{Phe} (data not shown). The rate constant of GTPase activation, k_3, and the rate of GTP hydrolysis, is increased more than 10-fold, to >500 s^{-1}, up to the level observed in the cognate situation without antibiotic[1] or with paromomycin (data not shown). As a consequence, the initial selection will be abolished in the presence of paromomycin, despite the decrease of k_{-2}.

The rate constant for the conformational switch of EF-Tu from the GTP to the GDP conformation, k_4, is decreased ~10-fold when paromomycin is present. The same decrease was observed in the cognate situation (data not shown). This effect suggests that the 30S subunit could influence EF-Tu, possibly by a direct contact (ref. 3) that is modulated by paromomycin binding. However, because the conformational change of EF-Tu is not rate limiting for the subsequent steps (Table 1), it has no relevance for selection. Functionally the most important effect of paromomycin is that it increases the accommodation rate constant, k_5, by a factor of 10 and, at the same time, decreases the rate constant of aa-tRNA rejection in the proofreading phase, k_7, about six-fold. As a result, the two rate constants are about equal, 0.9 and 1 s^{-1}, respectively. Thus, 50% of leucine is incorporated into dipeptide in the presence of paromomycin, about 30 times the level (1.5%) observed in the absence of antibiotic[2] (Fig. 3*d*). The increased level of leucine misincorporation induced by paromomycin under the present experimental conditions (no initial selection) is explained by both the failure of rejection and the acceleration of A site accommoda-

Table 2 Elemental rate constants of near-cognate EF-Tu•GTP•Leu-tRNA^{Leu2} binding to the A site

Step[1]		Rate constant (s^{-1})	
		-Paromomycin[2]	+Paromomycin
Initial binding	k_1	110 ± 20 (ftn. 3)	140 ± 20 (ftns 3,4)
	k_{-1}	25 ± 5	25 ± 10 (ftn. 4)
Codon recognition	k_2	100 ± 20	37 ± 2
	k_{-2}	17 ± 8	3.5 ± 0.5 (ftn. 5)
GTPase activation and GTP hydrolysis[6]	k_3	50 ± 20	>500 (ftn. 7)
GTP-GDP conf. change of EF-Tu	k_4	50 ± 20	6 ± 2
aa-tRNA accommodation and peptide bond formation[6]	k_5	0.1 ± 0.03	1 ± 0.1
Dissociation of EF-Tu•GDP	k_6	2 ± 1	ND[8]
aa-tRNA rejection	k_7	6 ± 1	0.9 ± 0.2

[1]Kinetic steps and rate constants are defined in Fig. 1.
[2]From ref. 2.
[3]μM^{-1} s^{-1}.
[4]Determined independently (Fig. 3*a*).
[5]Determined independently (Fig. 3*b*).
[6]Grouped for global analysis, because the former reaction is rate limiting[1,2].
[7]Rate too fast to be resolved.
[8]Not determined.

tion of near-cognate Leu-tRNA^{Leu2} during the proofreading step. It seems likely that the basic mechanism of aminoglycoside-induced misincorporation, as described here for Leu 2 on UUU codons and paromomycin, applies also to other near-cognate situations and other aminoglycosides, although there may be quantitative differences.

There are two possible ways to explain the effect of paromomycin in molecular terms. The antibiotic may directly interact with the anticodon region of the aa-tRNA and/or the mRNA[18] or, alternatively, it may induce a conformation of 16S rRNA such that 16S rRNA interactions with tRNA and/or mRNA are favored[7,12]. While both interpretations seem consistent with the observed stabilization by paromomycin of near-cognate aa-tRNA binding, the former does not explain the three-fold slower codon recognition observed in the presence of paromomycin. The former model is also difficult to reconcile with the acceleration observed for the forward reactions of GTPase activation and A site accommodation. Thus, the model involving a conformational switch of 16S rRNA seems more likely. The switch may extend beyond the decoding center, as recent X-ray crystallographic models of 30S ribosomal subunits[19] or of 70S ribosomes[20] suggest a close contact of the decoding region of 16S rRNA (helix 44) with helix 27 of 16S rRNA, another region that is involved in a switch regulating accuracy[5].

Interestingly, the same steps that are affected by paromomycin, that is, GTPase activation and A site accommodation, are much faster in the cognate situation than in the near-cognate one, suggesting an induced-fit mechanism of selection[2]. In order to rationalize induced fit in terms of structure, we suggest that the formation of the codon–anticodon complex induces a conformational transition of 16S rRNA that is similar to that caused by aminoglycoside binding and, in the cognate situation, is favored by structure-specific, sequence-independent interactions. Such contacts, which would increase the stability of the complex, could be formed between the A site 16S rRNA and the sugar-phosphate backbone of the codon–anticodon complex or residues of the codon–anticodon duplex that are in the same positions in all Watson–Crick base pairs. One prediction of the model is that codon recognition should be impaired when the antibiotic is bound. This is in fact observed, as the rate constant of codon recognition (k_2) is reduced about three-fold in the presence of paromomycin for both Leu 2 (Table 2) and Phe (data not shown). The model assumes that the conformational transition of 16S rRNA induced by cognate codon–anticodon complex formation is structurally coupled to the acceleration of GTPase activation and A site accommodation of aa-tRNA, although the mechanism of coupling is not clear at present. The near-cognate codon–anticodon complex would be less efficient in promoting the transition of 16S rRNA toward the productive conformation, and binding of paromomycin to the decoding center would be additionally required to induce the productive conformation of 16S rRNA.

Methods

Materials were prepared and GTP hydrolysis was measured as described[1,2]. EF-Tu•[γ-^{32}P]GTP•[^{14}C]Phe-tRNA^{Phe} and EF-Tu•[γ-^{32}P]GTP complexes were purified by gel filtration on Superdex 75 (Pharmacia). mRNAs (about 120 nucleotides long) contained similar ribosome binding sites and different codons following the AUG initiation codon (Table 1). mRNAs were prepared by runoff T7 RNA polymerase transcription of plasmids derived from plasmid pXR022 (ref. 21), provided by C. Gualerzi and R. Spurio (University of Camerino, Italy), or of plasmids coding for β-lactamase provided by G. Rauch (University of Witten, Germany). 70S initiation complexes with f[^{3}H]Met-tRNA^{fMet} in the P site were prepared in buffer A (50 mM Tris-HCl, pH 7.5; 70 mM NH_4Cl; 30 mM KCl; 7 mM $MgCl_2$, 1 mM dithiothreitol, DTT) as described[22]. Time courses of GTP hydrolysis (Fig. 2; Table 1) were measured at 20 °C either manually or by quench flow (Leu 2, Phe) with 0.1 μM EF-Tu•[γ-^{32}P]GTP•[^{14}C]Phe-tRNA^{Phe}; the concentration of initiation complexes usually was 0.3 μM, except for Leu 2 and Phe, where it was 1 μM.

Ribosomes programmed with poly(A), or with poly(U) carrying AcPhe-tRNA^{Phe} in the P site were prepared in buffer B (50 mM Tris-HCl, pH 7.5, 50 mM NH_4Cl, 10 mM $MgCl_2$, 1 mM DTT) and used for kinetic measurements of A site binding as described[1,2]. Rate constants were calculated from combined sets of time courses for proflavin fluorescence changes, GTP hydrolysis and dipeptide formation measured at five different ribosome concentrations. The data were globally fitted by numerical integration according to the mechanism of Fig. 1 using Scientist for Windows software (MicroMath Scientific Software, Salt Lake City)[1,2]. The fit yielded a unique solution for the rate constants k_2–k_7 as well as for the remaining fluorescence factors, provided that the values for k_1, k_{-1}, k_{-2}, and for the relative fluorescence of aa-tRNA in the initial binding complex were fixed. For k_5 and k_7, the same values were also obtained analytically. For values that were measured directly, standard deviations were calculated from the variation of several experiments. For values calculated by global fitting, the standard deviation for one parameter was determined for the case when all other parameters, except the ones measured directly, were allowed to change. That is, if a given parameter was set to a value outside the range of standard deviation, no fit satisfying all data sets could be obtained. This was particularly the case when the attempt was made to fit the data sets obtained in the presence of paromomycin using the parameters obtained in the absence of paromomycin.

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