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Nonenzymic Translocation and Spontaneous Release of Noncognate Peptidyl Transfer Ribonucleic Acid from *Escherichia coli* Ribosomes†

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ABSTRACT: Poly(uridylic acid)-programmed ribosomes have been used to synthesize the noncognate peptidyl-tRNA AcPhe-Tyr-tRNA^{Tyr} and its cognate counterpart Ac(Phe)$_2$-tRNA^{Phe}. After synthesis, Ac(Phe)$_2$-tRNA^{Phe} remains, as expected, in the ribosomal acceptor (A) site, but the noncognate AcPhe-Tyr-tRNA^{Tyr} does not; part of it spontaneously falls off the ribosome and the rest translocates, without elongation factor (EF) G, to the ribosomal donor site. The inhibitor of translocation viomycin prevents both the spontaneous release and the nonenzymatic translocation by confining the noncognate peptidyl-tRNA to the A site. Under these conditions, the interaction of AcPhe-Tyr-tRNA^{Tyr} with the A site appears to be similar to that of Ac(Phe)$_2$-tRNA^{Phe} without the antibiotic, and EF-G promotes the translocation and subsequent elongation of both peptidyl-tRNAs to comparable extents. The results indicate that, without viomycin, the noncognate peptidyl-tRNA is weakly held in the ribosomal A site and support the proposal that the release of peptidyl-tRNA occurring during protein synthesis in vivo is related to a ribosomal editing mechanism which discards mistranslated nascent proteins [Menninger, J. R. (1977) *Mech. Ageing Dev. 6*, 131].

Translocation is possibly the most complex step in the polypeptide chain elongation cycle. It comprises the coordinated movement of peptidyl-tRNA from the ribosomal acceptor (A)[1] site to the donor (P) site, the advance of mRNA in the length of three nucleotides (one codon), and the ejection of deacylated tRNA from the P site. Translocation requires the participation of elongation factor (EF) G and the hydrolysis of GTP [Modolell & Vázquez, 1975 (review)]. Little is known of the detailed molecular interactions and rearrangements of peptidyl-tRNA, mRNA, and ribosomal subunits that take place during translocation. One of the many unresolved problems is how the fidelity of base pairing between the mRNA and peptidyl-tRNA affects translocation. That base-pairing errors or distorted codon–anticodon interactions may alter the num-

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[1] Abbreviations used: A site, ribosomal acceptor site; P site, ribosomal donor site; EF-G, elongation factor G; EF-Tu, elongation factor Tu; EF-T, a mixture of EF-Tu and elongation factor Ts; AcPhe-tRNA^{Phe}, phenylalanine-specific *N*-acetylphenylalanyl transfer ribonucleic acid; AcPhe-Tyr-tRNA^{Tyr}, tyrosine-specific *N*-acetylphenylalanyltyrosyl transfer ribonucleic acid.

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ber of bases mRNA advances during each translocation step is suggested by the fact that the misreading-inducing antibiotic streptomycin phenotypically suppresses not only nonsense and missense but misreading-inducing frameshift mutations [Vãzquez, 1979 (review); Atkins et al., 1972], and that *strA* (*rpsL*) and *raml* (*rpsD*) mutants restrict and enhance, respectively, the frequency of nonsense, missense, and frameshift errors [see discussion by Kurland (1979)]. Moreover, base-pairing errors may have additional effects on translocation.

We have recently described a highly purified system which should facilitate study of the translocation of an incorrectly base-paired (noncognate) peptidyl-tRNA (Campuzano et al., 1979). It consists of *Escherichia coli* ribosomes complexed with poly(U) and AcPhe-tRNA^{Phe} in the P site, which bind Tyr-rRNA^{Tyr} and synthesize AcPhe-Tyr-tRNA^{Tyr}. The reaction is carried out in the presence of GTP, EF-Tu, and an antibiotic that induces misreading (streptomycin). With this system, we have found that the incorrectly base-paired AcPhe-Tyr-tRNA^{Tyr} [anticodon QUA; Gauss et al. (1979)] is unstable in the A site and is either released from the ribosome or translocated to the P site without the participation of EF-G.

Experimental Procedures

Materials. A preparation of EF-G, EF-T, and *Escherichia coli* MRE 600 ribosomes washed with 1 M NH_4Cl has been described elsewhere (Parmeggiani et al., 1971; Arai et al., 1972; Modolell & Vãzquez, 1973). Purified tRNA^{Phe} and tRNA^{Tyr} were obtained from Sigma Chemical Co. and were charged with [^{14}C]phenylalanine (980 cpm/pmol) and [^{3}H]-tyrosine (2200 cpm/pmol), respectively. Part of the [^{14}C]-Phe-tRNA^{Phe} was acetylated (Haenni & Chapeville, 1966). All labeled materials were from the Radiochemical Centre (Amersham). Sources of antibiotics have been previously described (Cabañas et al., 1978).

Binding of Ac[^{14}C]Phe-tRNA^{Phe} to Ribosomes. Ac[^{14}C]-Phe-tRNA^{Phe} was bound to poly(U)-programmed ribosomes either directly to the ribosomal P site (Tables II and III and Figures 2 and 3) or firstly to the A site and subsequently translocated in the presence of EF-G and GTP to the P site (Table I and Figure 1). Prior to the binding of Ac[^{14}C]-Phe-tRNA^{Phe}, poly(U) was bound to ribosomes in mixtures (120 μL) containing 90 mM NH_4Cl, 17 mM Tris-HCl, pH 7.8, 10 mM magnesium acetate, 2.5 mM dithiothreitol, 0.16 mg/mL poly(U), and 89 A_{260} units/mL ribosomes. Incubation was at 30 °C for 3 min. To bind Ac[^{14}C]Phe-tRNA^{Phe} directly to the P site, the above mixture was supplemented with this compound and sufficient salts to yield the following mixture (240 μL): 45 mM NH_4Cl, 25 mM KCl, 21 mM Tris-HCl, pH 7.8, 8 mM magnesium acetate, 2.5 mM dithiothreitol, 0.08 mg/mL poly(U), 45 A_{260} units/mL ribosomes, and 0.55 μM Ac[^{14}C]Phe-tRNA^{Phe}. After incubation at 30 °C for 30 min, the mixture was passed through a Sepharose 6B column (0.6 × 15 cm) equilibrated with 70 mM NH_4Cl, 8 mM magnesium acetate, 20 mM Tris-HCl, pH 7.8, and 6 mM 2-mercaptoethanol to remove unbound Ac[^{14}C]Phe-tRNA^{Phe} and poly(U). Fractions containing most ribosomes were pooled (0.5 mL) and used immediately to bind aminoacyl-tRNA to the ribosomal A site. These ribosomes contained 4–7 pmol of bound Ac[^{14}C]Phe-tRNA^{Phe} per A_{260} unit of ribosomes, more than 90% of this ligand being reactive with puromycin (Leder & Bursztyn, 1966).

Binding of Ac[^{14}C]Phe-tRNA^{Phe} to the ribosomal A site was performed in the presence of 20 mM magnesium acetate and deacylated tRNA^{Phe} as previously described (Modolell et al., 1973). Translocation of Ac[^{14}C]Phe-tRNA^{Phe} to the ribosomal P site was carried out in mixtures (0.33 mL) containing 20 mM NH_4Cl, 40 mM KCl, 12.5 mM magnesium acetate, 19 mM Tris-HCl, pH 7.8, 1.7 mM dithiothreitol, 0.12 mM GTP, 40 μg/mL EF-G, and 29 A_{260} units/mL ribosomes complexed with poly(U) and 6–9 pmol of Ac[^{14}C]Phe-tRNA^{Phe} per A_{260} unit of ribosomes. After incubation at 30 °C for 10 min, the mixture was passed through a Sepharose 6B column equilibrated with buffer of the same ionic composition as the translocation mixture and the fractions containing most of the ribosomes were collected and used immediately.

Binding of Tyr-tRNA^{Tyr} or Phe-tRNA^{PhE} to Ribosomes and Release of AcPhe-(aminoacyl)$_n$-tRNA. Binding of [^{3}H]-Tyr-tRNA^{Tyr} or [^{3}H]Phe-tRNA^{Phe} to ribosomes complexed with poly(U) and P-site-bound Ac[^{14}C]Phe-tRNA^{Phe} was performed in mixtures (160–250 μL) containing, unless otherwise specified, either 70 mM NH_4Cl or 30 mM NH_4Cl plus 40 mM KCl, 10 mM magnesium acetate, 10–20 mM Tris-HCl, pH 7.8, 5 mM 2-mercaptoethanol, 0–2 mM dithiothreitol, 4 μM streptomycin, 0.25–0.35 μM of either [^{3}H]-Tyr-tRNA^{Tyr} or [^{3}H]Phe-tRNA^{Phe}, 10 μg/mL EF-T, 0.1 mM GTP, and 8 A_{260} units/mL ribosomes containing 4–9 pmol of P-site-bound Ac[^{14}C]Phe-tRNA^{Phe} per A_{260} unit of ribosomes. (In the experiments described in Tables II and III, GTP (0.8 mM) was preincubated at 30 °C for 10 min, in the presence of 10 mM phosphoenol pyruvate and 0.2 mg/mL pyruvate kinase under the appropriate ionic conditions, to remove contaminating GDP.) After incubation at 30 °C for 10 min, portions (15–30 μL) were withdrawn to determine the amount of ^{14}C- and ^{3}H-labeled acylated tRNA bound to ribosomes. The samples were diluted with 1–2 mL of buffer with the same composition as the reaction mixture and filtered through nitrocellulose membranes (Millipore HA). Without being washed, the filters were freed of excess buffer with the help of blotting paper and then dried and counted while being immersed in a toluene-based scintillation fluid. Larger samples (100–150 μL) from the binding reaction were used to determine the amount of ^{14}C-labeled products bound and released from ribosomes and the composition of both types of products. The samples were diluted with 1.5 or 2 mL of buffer and filtered through two nitrocellulose membranes. The filters, without being washed, were processed as above and counted. The recovery of ^{14}C in the lower filter was between 10 and 40% of that in the upper filter. Most of the counts in both filters were due to ribosome-bound products, since controls without ribosomes showed that Ac[^{14}C]Phe-tRNA was retained by filters only in the amount expected from the volume of buffer held up by the filters (approximately 50 μL/filter). For correction for the ^{14}C radioactivity not bound to ribosomes and contained in the 0.1 mL of diluted reaction mixture wetting the filters, the filtrates were collected and their ^{14}C radioactivity was determined by mixing 0.30–0.40-mL portions with 3–4 mL of Bray's scintillation fluid and counting. The radioactivity corresponding to 0.1 mL of the filtrate was calculated, corrected by the different counting efficiencies in Bray's (37%) fluid and on Millipore filters (64%), and subtracted from the ^{14}C radioactivity found on the filters. The amount of ^{14}C-labeled material released from ribosomes during the binding of [^{3}H]aminoacyl-tRNA was determined by calculating the radioactivity corresponding to the total volume of diluted binding mixture (1.6–2.15 mL).

Electrophoretic Analysis of Ac[^{14}C]Phe-(aminoacyl)$_n$-tRNA. The composition of ribosome-bound and -released ^{14}C-labeled products formed during the binding of either [^{3}H]Phe-tRNA^{Phe} or [^{3}H]Tyr-tRNA^{Tyr} to Ac[^{14}C]Phe-tRNA^{Phe}·ribosome·poly(U) complexes was determined by

Table I: Binding of either [^{3}H] Phe-tRNA^{Phe} or [^{3}H] Tyr-tRNA^{Tyr} to Ac[^{14}C] Phe-tRNA^{Phe}·Ribosome·Poly(U) Complexes and Reaction with Puromycin of the Bound Products[a]

expt	molecules per ribosome			Ac[^{14}C] Phe-Pur (%)	Ac[^{14}C] Phe-$(AA)_{n\geq 1}$-Pur (%)
	Ac[^{14}C] Phe-tRNA	[^{3}H] Phe-tRNA	[^{3}H] Tyr-tRNA		
1, bound to ribosomes	0.24	0.28			
puromycin reactive	0.06	0.05			
puromycin reactive + EF-G	0.24	0.59		16	84
2, bound to ribosomes	0.17		0.22		
puromycin reactive	0.16		0.14		
puromycin reactive + EF-G	0.16		0.16	57	43

[a] Binding of either [^{3}H] Phe-tRNA^{Phe} or [^{3}H] Tyr-tRNA^{Tyr} to ribosomes carrying Ac[^{14}C] Phe-tRNA^{Phe} in the P site was performed in parallel reaction mixtures as described under Experimental Procedures. After binding was complete, duplicate 30-μL portions of each reaction mixture were withdrawn and used to determine the amount of ribosome-bound ^{14}C and ^{3}H radioactivity (Nirenberg & Leder, 1964). Other 40-μL portions of the binding mixtures were supplemented with 0.5 mM puromycin, incubated at 30 °C for 10 min, and analyzed for Ac[^{14}C] Phe-([^{3}H] aminoacyl)$_{n\geq 0}$-puromycin synthesized, as described below. Still other 100-μL portions were supplemented with 0.5 mM puromycin, 0.1 mM GTP, and 40 μg/mL EF-G and incubated at 30 °C for 10 min, and the reaction was stopped with 250 μL of 0.1 M sodium acetate, pH 5.0. The Ac[^{14}C] Phe-([^{3}H] aminoacyl)$_{n\geq 0}$-puromycin synthesized was extracted with 0.65 mL of ethyl acetate; 0.2 mL of the organic phase was removed and counted and, to analyze the products of the reaction, 0.4 mL was spotted on Whatman No. 1 paper strips and subjected to electrophoresis for 1.5 h at 60 V/cm in 0.05 M pyridine acetate, pH 3.5. The strips were cut and counted as described under Experimental Procedures. Ac[^{14}C] Phe-puromycin moved 8 cm toward the cathode and Ac[^{14}C] Phe-([^{3}H] aminoacyl)$_{n\geq 1}$-puromycin between 0 and 5 cm in the same direction. One hundred percent represents 950 and 740 cpm of ^{14}C radioactivity recovered from the electropherograms in the experiments performed with [^{3}H] Phe-tRNA^{Phe} and [^{3}H] Tyr-tRNA^{Tyr}, respectively.

paper electrophoresis. The pair of Millipore filters containing the ribosome-bound products was removed from the counting fluid (no appreciable radioactivity remained in the fluid), rinsed with toluene to eliminate the scintillator, dried, placed in screw-caped vials, and twice extracted with 1 mL of 1 M NH_4OH at room temperature for 20 min, with shaking. This procedure normally extracted over 90% of the radioactivity in the filters; however, when a large number of long polypeptide chains were present (i.e., when the reaction mixture contained Phe-tRNA^{Phe}, EF-G, and GTP), efficiency decreased to approximately 70%. The extracts were pooled and evaporated to dryness in a rotary evaporator under vacuum. The residue was taken up with 0.4 mL of 0.5 M NaOH and was incubated at 37 °C for 1 h to hydrolyze the tRNA portion of the products. The solution was acidified with 25 μL of concentrated HCl and extracted with 1 mL of ethyl acetate. The organic phase (0.8 mL) was evaporated to dryness under an air stream, and the residue was dissolved in 25 μL of phenol saturated with H_2O; 20 μL was spotted on Whatman No. 1 paper strips, and 2 μL was mixed with Bray's scintillation fluid and counted. Electrophoresis of the strips was for 1 h at 55 V/cm in 0.1 M ammonium acetate (pH 6.9). The strips were dried and cut in 1-cm pieces, which were counted while being immersed in a toluene-based scintillation fluid. Under the indicated conditions, migrations toward the anode were the following: AcPhe, 25 cm; $Ac(Phe)_2$ or AcPhe-Tyr, 17 cm; $Ac(Phe)_3$ or AcPhe-$(Tyr)_2$, 14 cm; $Ac(Phe)_4$ or AcPhe-$(Tyr)_3$, 11 cm; larger oligopeptides or polypeptides remained at or close to the origin. Recovery of ^{14}C radioactivity in the electrophoretic analysis was between 87 and 99% of the radioactivity placed on the strips.

For determination of the composition of the Ac[^{14}C] Phe-(aminoacyl)$_n$-tRNA released from ribosomes, 1150 μL of the filtrate from the diluted binding mixture was mixed with 125 μL of 5 M NaOH. After incubation at 37 °C for 1 h, the hydrolysate was acidified and ethyl acetate extracted and analyzed by paper electrophoresis as described above.

Results

Nonenzymic Translocation of Noncognate Peptidyl-tRNA. Our previous work has shown that aminoglycoside antibiotics that induce misreading promote the efficient binding of Tyr-tRNA^{Tyr} to the A site of poly(U)-programmed ribosomes and that, when AcPhe-tRNA^{Phe} is present in the P site, Tyr-tRNA^{Tyr} forms a dipeptide with its AcPhe portion (Campuzano et al., 1979). We have now examined the translocation of the synthesized noncognate AcPhe-Tyr-tRNA^{Tyr} and compared it with that of the cognate Ac-$(Phe)_2$-tRNA^{Phe}. The experiments were performed in the presence of streptomycin, and although this antibiotic appears to have a minimal effect on translocation (Cabañas et al., 1978), we first verified that it does not affect the translocation of the cognate Ac[^{14}C]Phe-[^{3}H]Phe-tRNA^{Phe}. This compound was synthesized on ribosomes in the presence of streptomycin and, as expected, was located in the ribosomal A site since most of it did not react with puromycin (Table I; compare lines 1 and 2). Addition of EF-G promoted its translocation, as indicated by the quantitative reaction of the ribosome-bound ^{14}C-labeled material with puromycin (Table I, lines 1 and 3). Moreover, analysis of the puromycin-containing product showed that most of it was Ac[^{14}C]Phe-([^{3}H]Phe)$_{n\geq 1}$-puromycin (Table I, line 3, column 5). It should be noted that, in the presence of EF-G, the amount of [^{3}H]Phe reacted with puromycin was much higher than the amount of [^{3}H]Phe-tRNA^{Phe} initially bound to the ribosome (0.59 vs. 0.28 molecules/ribosome, Table I), probably because the factor promoted elongation of the dipeptidyl chains before they reacted with puromycin.

Ac[^{14}C]Phe-[^{3}H]Tyr-tRNA^{Tyr} was synthesized under the same conditions as its cognate counterpart. However, Table I (experiment 2) shows that in the absence of EF-G this noncognate peptidyl-tRNA reacted quantitatively with puromycin and that addition of the factor did not further increase the reaction. Moreover, the possibility that Ac[^{14}C]Phe-tRNA^{Phe} and [^{3}H]Tyr-tRNA^{Tyr} failed to form a dipeptide on most active ribosomes and that puromycin reacted separately with each of these ligands was ruled out by analyzing the product of the reaction; nearly half of this was Ac[^{14}C]-Phe-([^{3}H]Tyr)$_{n\geq 1}$-puromycin (Table I, line 6, column 5). These results therefore suggested that AcPhe-Tyr-tRNA^{Tyr} was not retained in the A site after its synthesis but moved, without the participation of EF-G, to the ribosomal P site.

It should be stressed that it seems very unlikely that this nonenzymatic translocation of AcPhe-Tyr-tRNA^{Tyr} would in fact result from contamination of our system with EF-G, since the cognate $Ac(Phe)_2$-tRNA^{Phe} in the parallel binding mixture

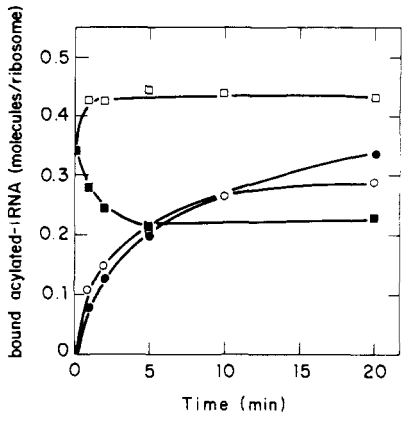

FIGURE 1: Effect of binding to the ribosomal A site of either [^{3}H]-Phe-tRNA^{Phe} or [^{3}H]Tyr-tRNA^{Tyr} on the retention by ribosomes of P-site-bound Ac[^{14}C]Phe-tRNA^{Phe}. [^{3}H]Aminoacyl-tRNA was bound to Ac[^{14}C]Phe-tRNA^{Phe}·ribosome·poly(U) complexes as described under Experimental Procedures, except that 30-μL portions of the reaction mixture were withdrawn at the indicated times and analyzed for bound acylated-tRNA. (○) Binding of [^{3}H]Phe-tRNA^{Phe}; (●) binding of [^{3}H]Tyr-tRNA^{Tyr}; (□ or ■) ^{14}C label remaining on ribosomes after binding [^{3}H]Phe-tRNA^{Phe} or [^{3}H]Tyr-tRNA^{Tyr} for the indicated time.

did not translocate unless EF-G was added (Table I). Moreover, EF-T, which was homogeneous as judged by polyacrylamide gel electrophoresis in sodium dodecyl sulfate, and ribosome preparations did not contain EF-G since thiostrepton-sensitive GTP hydrolysis was undetectable when both preparations were mixed [not shown; see Modolell & Vázquez (1975)]. Finally, it should be indicated that although the ribosomes used in the experiments represented in Table I had been exposed to EF-G during the binding of AcPhe-tRNA^{Phe} and the factor had subsequently been removed by gel filtration, the nonenzymatic translocation was also observed with ribosomes that bound AcPhe-tRNA^{Phe} directly to the P site and, therefore, had not been exposed to EF-G (not shown).

Spontaneous Release of Noncognate Peptidyl-tRNA from Ribosomes. The binding of [^{3}H]Tyr-tRNA^{Tyr} to ribosomes complexed with Ac[^{14}C]Phe-tRNA^{Phe} decreased the amount of ^{14}C-labeled material retained by these ribosomes (Table I; compare lines 1 and 4). This effect is shown in more detail in Figure 1, which illustrates the time courses of [^{3}H]Tyr-tRNA^{Tyr} and [^{3}H]Phe-tRNA^{Phe} binding to ribosomes and the retention of ^{14}C radioactivity. The release of part of the ^{14}C-labeled material was associated only with the binding of the noncognate substrate.

The composition of the ribosome-bound and -released ^{14}C-labeled materials after the binding of [^{3}H]Tyr-tRNA^{Tyr} was examined. The binding mixture was filtered through nitrocellulose membranes and the ribosome-bound and -released products were recovered from the filter and the filtrate, respectively. The released material was precipitable by cold 10% trichloroacetic acid but was soluble in this acid at 90 °C (not shown). Its hydrolysate was analyzed by paper electrophoresis, and Figure 2 shows that most of the labeled residue was Ac[^{14}C]Phe-[^{3}H]Tyr. These results suggest that the product released from ribosomes was Ac[^{14}C]Phe-[^{3}H]Tyr-tRNA^{Tyr}.

The ribosome-bound material was similarly analyzed. The electrophoretic analysis of its alkaline hydrolysate indicated that nearly half of it was Ac[^{14}C]Phe-([^{3}H]Tyr)$_{n\geq 1}$-tRNA^{Tyr}, the remainder being Ac[^{14}C]Phe-tRNA^{Phe} (Figure 2). Moreover, more than one-third of the Ac[^{14}C]Phe-([^{3}H]-Tyr)$_{n\geq 1}$-tRNA^{Tyr} had two or more tyrosyl residues, thus demonstrating that a substantial part of the population of ribosome-bound peptidyl-tRNA molecules could undergo more than one round of nonenzymatic translocation.

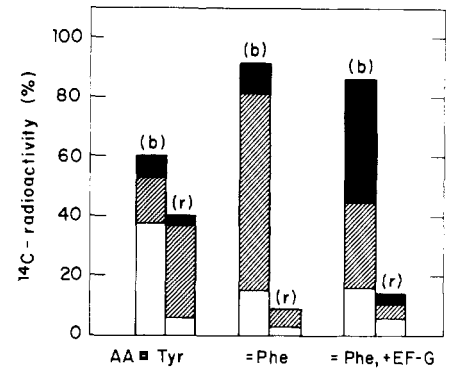

FIGURE 2: Ribosome-bound (b) and -released (r) Ac[^{14}C]Phe-([^{3}H]aminoacyl)$_{n\geq 0}$-tRNA after the binding of either [^{3}H]Tyr-tRNA^{Tyr} or [^{3}H]Phe-tRNA^{Phe} to Ac[^{14}C]Phe-tRNA^{Phe}·ribosome·poly(U) complexes. [^{3}H]Aminoacyl-tRNA was bound to the A site of ribosomal complexes and the ribosome-bound and -released products were analyzed as described in the text, except that after binding was complete, one 150-μL portion of the mixture for [^{3}H]Phe-tRNA^{Phe} binding was made 0.1 mM in GTP and 30 μg/mL in EF-G, and the incubation was continued at 30 °C for 5 min before analyzing the products. One hundred percent represents the sum of ^{14}C radioactivity retained by the Millipore filters plus that found in the filtrates (4280–4440 cpm), which amounts to 97–100% of the ^{14}C radioactivity placed in the reaction mixtures. (□) Ac[^{14}C]Phe; (▨) Ac[^{14}C]-Phe-[^{3}H]AA; (■) Ac[^{14}C]Phe-([^{3}H]AA)$_{n\geq 1}$.

The composition of the ribosome-bound and -released ^{14}C-labeled materials following the binding of [^{3}H]Phe-tRNA^{Phe} was also examined (Figure 2). Over 90% of the ^{14}C label remained bound to ribosomes and consisted mainly of Ac-[^{14}C]Phe-[^{3}H]Phe-tRNA^{Phe}; only 13% of this dipeptide underwent further elongation. Moreover, the addition of EF-G promoted the translocation and elongation, but not the release, of this cognate peptidyl-tRNA. Thus, the great extent of spontaneous release and nonenzymatic translocation is characteristic of the noncognate peptidyl-tRNA.

Effect of Viomycin and Other Antibiotics on the Spontaneous Release and Nonenzymatic Translocation of Noncognate Peptidyl-tRNA. Viomycin is a well-characterized inhibitor of translocation that interferes with this process by preventing the removal of peptidyl-tRNA from the A site (Modolell & Vázquez, 1977). Table II shows that viomycin strongly inhibited the release of noncognate peptidyl-tRNA and promoted the accumulation of Ac[^{14}C]Phe-[^{3}H]Tyr-tRNA^{Tyr} on the ribosomes. In spite of such an accumulation, only negligible amounts of tripeptides and larger peptides were synthesized, indicating that viomycin also inhibited nonenzymatic translocation. The peptidyl transferase inhibitors streptogramin A and sparsomycin (Vázquez, 1979) also impaired the release of ^{14}C-labeled material from ribosomes, but their effect was probably due to the inhibition of the synthesis of the noncognate Ac[^{14}C]Phe-[^{3}H]Tyr-tRNA^{Tyr} (Table II).

The effect of viomycin was further investigated. This antibiotic strengthens the binding of peptidyl-tRNA to the ribosomal A site, but it does not block the interaction of EF-G with the ribosome (Modolell & Vázquez, 1977). Thus, we reasoned that viomycin might strengthen the binding of noncognate peptidyl-tRNA to the A site to a degree sufficient to block the nonenzymatic translocation and spontaneous release (Table II) but insufficient to prevent translocation in the presence of EF-G and GTP. Table III, lines 1 and 2, shows that this was indeed the case. EF-G promoted extensive elongation of the dipeptidyl-tRNA so that the 5% of tetrapeptides and longer peptides found without the factor was increased to 37%. On the other hand, with the cognate Ac-

Table II: Inhibition by Antibiotics (10 μM) of the Release of Ac[^{14}C]Phe-([^{3}H]Tyr)$_{n\geq 1}$-tRNA^{Tyr} from Ribosomes: Composition of the Ribosome-Bound Peptidyl-tRNA[a]

antibiotic	released peptidyl-tRNA	ribosome-bound ^{14}C radioactivity (%) in Ac[^{14}C]Phe-tRNA	Ac[^{14}C]Phe-[^{3}H]Tyr-tRNA	Ac[^{14}C]Phe-([^{3}H]Tyr)$_{n\geq 1}$-tRNA
none	34	46	9	11
viomycin	6	33	55	6
streptogramin A	12	83	1	4
sparsomycin	9	ND[b]	ND	ND

[a] Binding of [^{3}H]Tyr-tRNA^{Tyr} to Ac[^{14}C]Phe-tRNA^{Phe}·ribosome·poly(U) complexes, in the presence of the indicated antibiotics, was as described under Experimental Procedures. Release of Ac[^{14}C]Phe-([^{3}H]Tyr)$_{n\geq 1}$-tRNA^{Tyr} was measured by the decrease in ^{14}C radioactivity that was retained by Millipore filters. The peptidyl-tRNA that remained on the filters was extracted and analyzed as described in the text. One hundred percent represents 4570 cpm of ^{14}C radioactivity, equivalent to 0.20 molecule of peptidyl-tRNA per ribosome. [b] ND = not determined.

Table III: Effect of Viomycin and EF-G on Oligopeptide Synthesis on Ac[^{14}C]Phe-tRNA^{Phe}·Ribosome·Poly(U) Complex Promoted by either [^{3}H]Tyr-tRNA^{Tyr} or [^{3}H]Phe-tRNA^{Phe}[a]

additions	AA-tRNA used	Ac[^{14}C]Phe-([^{3}H]AA)$_n$ (%) n = 1	n = 2	n = 3	n > 3
viomycin	[^{3}H]Tyr-tRNA^{Tyr}	93	2		5
viomycin + EF-G	[^{3}H]Tyr-tRNA^{Tyr}	45	18	16	21
viomycin	[^{3}H]Phe-tRNA^{Phe}	97	1		2
viomycin + EF-G	[^{3}H]Phe-tRNA^{Phe}	69	19	8	4
EF-G	[^{3}H]Phe-tRNA^{Phe}	40	11	6	43

[a] Binding of either [^{3}H]Tyr-tRNA^{Tyr} or [^{3}H]Phe-tRNA^{Phe} to Ac[^{14}C]Phe-tRNA·ribosome·poly(U) complexes was performed as described under Experimental Procedures, except that streptomycin was omitted and 10 μM viomycin was added when indicated, since it was found that viomycin alone was capable of inducing the binding of noncognate Tyr-tRNA^{Tyr} to poly(U)-programmed ribosomes. After binding was complete, 30 μg/mL EF-G was added to 120-μL portions of the binding mixtures and incubation at 30 °C was continued for 10 min; 100-μL portions of every mixture were then withdrawn and analyzed by paper electrophoresis for oligopeptides synthesized (Experimental Procedures). Between 70% and 87% of the Ac[^{14}C]Phe-tRNA^{Phe} present on ribosomes at the start of the binding reaction was converted to oligopeptides. One hundred percent represents 1170–1580 cpm of ^{14}C radioactivity in oligopeptides recovered from the electropherograms. Results similar to those in this table were obtained in a parallel experiment performed in the presence of both streptomycin and viomycin (not shown).

(Phe)$_2$-tRNA^{Phe}, which has a higher affinity for the A site, EF-G in the presence of the antibiotic induced a much lower extent of polymerization (12% of tetrapeptides and longer peptides; Table III, lines 3 and 4). Moreover, the extent of polymerization observed with noncognate AcPhe-Tyr-tRNA^{Tyr} and viomycin plus EF-G is comparable to that found with the cognate Ac(Phe)$_2$-tRNA^{Phe} and EF-G, but without viomycin (37% and 49% of chains equal to or longer than tetrapeptides, respectively).

Figure 3 shows the kinetics of translocation, as measured by the puromycin reaction, of the cognate and noncognate Ac[^{14}C]Phe-[^{3}H]aminoacyl-tRNA in the presence of viomycin and EF-G. Most of the noncognate substrate reacted within 2 min with puromycin, and a plateau was reached after 4 min. Very similar kinetics have previously been observed in our laboratory for the translocation of AcPhe-tRNA^{Phe} in the absence of viomycin [Figure 1 of Modolell et al. (1973)]. In contrast, the translocation of the cognate peptidyl-tRNA in the presence of viomycin was one order of magnitude slower than that of the noncognate substrate.

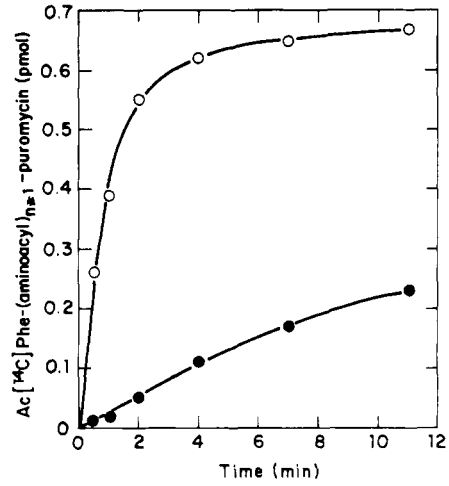

FIGURE 3: Effect of viomycin on the translocation of Ac[^{14}C]Phe-[^{3}H]Tyr-tRNA^{Tyr} (○) and of Ac[^{14}C]Phe-[^{3}H]Phe-tRNA^{Phe} (●). [^{3}H]Tyr-tRNA^{Tyr} or [^{3}H]Phe-tRNA^{Phe} was bound to Ac[^{14}C]Phe-tRNA^{Phe}·ribosome·poly(U) complexes as described under Experimental Procedures except that streptomycin was replaced by 10 μM viomycin. After binding was complete, the reaction mixtures (200 μL) were cooled to 0 °C, supplemented with 25 μg/mL EF-G and 0.45 mM puromycin, and incubated at 30 °C. After the indicated time intervals, 30-μL portions containing 5.3 pmol of ribosomes were withdrawn and assayed for Ac[^{14}C]Phe-(aminoacyl)$_{n\geq 1}$-puromycin synthesized, essentially as described in the legend to Table I. At the start of the puromycin reaction, 0.76 and 0.93 pmol of ^{14}C-labeled product were bound to ribosomes per 30-μL sample in the reaction with [^{3}H]-Tyr-tRNA^{Tyr} and [^{3}H]Phe-tRNA^{Phe}, respectively. The amount of ^{3}H-labeled product bound to ribosomes was 3.2 pmol in both cases.

Discussion

AcPhe-Tyr-tRNA^{Tyr} is weakly retained by the A site of poly(U)-programmed ribosomes, and, after its synthesis, part of it (60%) is released from the ribosomes (Figure 2) while the rest translocates to the P site (Table I). However, only approximately one-third of the translocated AcPhe-Tyr-tRNA^{Tyr} molecules seems to have effected bona fide nonenzymatic translocations, since only this fraction is capable of undergoing further elongation (Figure 2). Thus, the mechanism of this nonenzymatic translocation is not clearly understood. Nevertheless, it is probably not mediated by the release of the noncognate peptidyl-tRNA and its subsequent binding to ribosomes with vacant P sites. We have found (not shown) that excess vacant ribosomes do not increase the amount of AcPhe-Tyr-tRNA^{Tyr} reactive with puromycin and that AcTyr-tRNA^{Tyr} does not bind to the puromycin-reactive site of poly(U)-programmed ribosomes. It is also unknown whether this nonenzymatic translocation occurs by a mechanism similar to that of the slow, *p*-(chloromercuri)benzoate-stimulated nonenzymatic translocation of (Phe)$_n$-tRNA^{Phe} (Pestka, 1968; Gavrilova & Spirin, 1971; Gavrilova et al., 1974).

In contrast to the weak binding of AcPhe-Tyr-tRNA^{Tyr},

Tyr-tRNA^{Tyr} is stable in the ribosomal A site (Campuzano et al., 1979). Since, barring conformational differences, the only structural difference between Tyr-tRNA^{Tyr} and AcPhe-Tyr-tRNA^{Tyr} resides in the aminoacyl residues, the different stability of the two ligands may be due to a stronger interaction of Tyr-tRNA^{Tyr} with the ribosomal region normally in contact with the aminoacylated end of tRNA, the peptidyltransferase center. This suggestion is supported by the observed preferential binding of aminoacyl oligonucleotides to the A site of peptidyltransferase (Celma et al., 1971) and of N-acylated aminoacyl oligonucleotides to the P site of the enzyme (Celma et al., 1970). It should be noted that, during protein synthesis, a decrease of the affinity of the acylated tRNA in the A site subsequent to peptidyl transfer may facilitate its translocation to the P site.

It has been shown that peptidyl-tRNA occasionally dissociates from the ribosome during protein synthesis in vivo (Menninger, 1976) and that amino acid starvation differentially enhances the dissociation of specific sets of peptidyl-tRNA families (Caplan & Menninger, 1979). These observations have led to the proposal of a ribosomal mechanism by which noncognate peptidyl-tRNAs are recognized, released from the ribosome, and subsequently degraded to prevent completion and accumulation of mistranslated proteins (Menninger, 1977). Our finding that AcPhe-(Tyr)$_{n\geq 1}$-tRNA^{Tyr}, but not its cognate counterpart (Figure 2), is released from ribosomes in vitro suggests that such an editing mechanism might be based on a decreased affinity of the ribosomal A site for peptidyl-tRNAs with erroneous codon–anticodon interactions. Correct codon–anticodon interactions are known to promote conformational modifications of the tRNA molecule (Wagner & Garrett (1979) and references therein). Thus, it is conceivable that incorrect interactions might be unable to induce the conformational changes necessary to stabilize the binding of the noncognate peptidyl-tRNA to the A site, thereby facilitating its release.

Viomycin is an inhibitor of translocation which prevents the exit of peptidyl-tRNA from the A site (Modolell & Vázquez, 1977). This antibiotic inhibits both the spontaneous release and nonenzymatic translocation of noncognate peptidyl-tRNA (Table II) and, therefore, probably increases the affinity of this ligand for the A site. Moreover, in the presence of EF-G, viomycin inhibits translocation of cognate peptidyl-tRNA but not that of noncognate AcPhe-(Tyr)$_{n\geq 1}$-tRNA^{Tyr} (Table III, Figure 3). These results suggest that most of the effects of viomycin of the elongation cycle can be explained by an increase of the affinity of the A site for acylated tRNA. With cognate peptidyl-tRNA, which binds strongly to the A site, viomycin reinforces the interaction, leading to the inability of EF-G to remove it from the A site (inhibition of translocation). With noncognate peptidyl-tRNA, which is weakly held in the A site, the increased affinity stabilizes the binding (thus preventing the spontaneous release and nonenzymatic translocation), but to a lesser extent than that of cognate peptidyl-tRNA; consequently, EF-G can promote translocation of the noncognate substrate. Moreover, viomycin may also increase the affinity of the A site for aminoacyl-tRNA since we have found that it induces the binding of Tyr-tRNA^{Tyr} to poly(U)-programmed ribosomes (misreading; see legend to Table III and P. Marrero, M. J. Cabañas, and J. Modolell, unpublished results).

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