Ribosome Protection by tRNA Derivatives against Inactivation by Virginiamycin M: Evidence for Two Types of Interaction of tRNA with the Donor Site of Peptidyl Transferase†

G. Chinali,‡ M. Di Giambattista,§ and C. Cocito*,§

Istituto di Scienze Biochimico-Fisiche, IIa Facoltà di Medicina e Chirurgia, Università di Napoli, Napoli, Italy, and Department of Microbiology and Genetics, Institute of Cell Pathology, University of Louvain Medical School, Brussels 1200, Belgium

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ABSTRACT: Virginiamycin M (VM) was previously shown to interfere with the function of both the A and P sites of ribosomes and to inactivate tRNA-free ribosomes but not particles bearing peptidyl-tRNA. To explain these findings, the shielding ability afforded by tRNA derivatives positioned at the A and P sites against VM-produced inactivation was explored. Unacylated $tRNA^{Phe}$ was ineffective, irrespective of its position on the ribosome. Phe-tRNA and Ac-Phe-tRNA provided little protection when bound directly to the P site but were active when present at the A site. Protection by these tRNA derivatives was markedly enhanced by the formation of the first peptide bond and increased further upon elongation of peptide chains. Most of the shielding ability of Ac-Phe-tRNA and Phe-tRNA positioned at the A site was conserved when these tRNAs were translocated to the P site by the action of elongation factor G and GTP. Thus, a 5–10-fold difference in the protection afforded by these tRNAs was observed, depending on their mode of entry to the P site. This indicates the occurrence of two types of interaction of tRNA derivatives with the donor site of peptidyl transferase: one shared by acylated tRNAs directly bound to the ribosomal P site (no protection against VM) and the other characteristic of aminoacyl- or peptidyl-tRNA translocated from the A site (protection of peptidyl transferase against VM). To explain these data and previous observations with other protein synthesis inhibitors, a new model of peptidyl transferase is proposed. This model postulates the existence of two equivalent substrate binding sites exchanging their position, conformation, and function during translocation, which would require no detachment of peptidyl-tRNA from peptidyl transferase.

A unique property of virginiamycin-like antibiotics (synergimycins) is the presence of two components, type A and B, which act synergistically on bacteria: single components produce a bacteriostatic effect, whereas their mixture is bactericidal [cf. the review articles of Vazquez (1967, 1975), Tanaka (1975), Cocito (1979, 1983), and Cocito and Chinali (1985)]. Both A and B components inhibit protein synthesis in vivo and form 1:1 complexes with the 50S ribosomal subunit in vitro (Cocito, 1969; Ennis, 1971, 1974; Contreras & Vazquez, 1977). Virginiamycin M (VM,[1] a type A component) produces a lasting alteration of ribosomes in vitro: after a transient interaction with VM, followed by its removal, ribosomes are inactive in promoting peptide bond formation in cell-free systems (Parfait & Cocito, 1980). This effect is secondary to the production of a stable conformational change of the ribosome (Moureau et al., 1983a,b; Di Giambattista et al., 1984), which involves a permanent alteration of both acceptor and donor sites of peptidyl transferase (Chinali et al., 1981, 1984). Inactivation of the substrate acceptor site of peptidyl transferase is responsible for the previously observed inhibition of aminoacyl-tRNA enzymatic binding to ribosomes (Cocito & Kaji, 1971; Cocito et al., 1974) whereas an alteration of the donor site accounts for the block of peptidylpuromycin formation (Cocito & Kaji, 1971; Cocito et al., 1974; Monro & Vazquez, 1967). When ribosomes are already engaged in peptide chain elongation, however, a reduced activity of the antibiotic is observed (Contreras & Vazquez, 1977; Chinali et al., 1981).

The aim of this work was to explore the mechanism of this phenomenon. The approach chosen was to test the ability of various tRNA derivatives bound either enzymatically or nonenzymatically to the ribosomal A or P sites to protect peptidyl transferase against inactivation by VM. Moreover, the shielding capacity of different tRNAs positioned at the P site was tested after their direct binding to this site or after their translocation from the A site.

Materials and Methods

Biochemicals. NH_4Cl-washed ribosomes, IF-1, -2, and -3, phenylalanyl-tRNA synthetase, EF-G, EF-Tu, and EF-Ts were prepared from *Escherichia coli* as described (Cocito et al., 1974; Chinali et al., 1981; Chinali & Parmeggiani, 1982). Aminoacylation of $tRNA^{Phe}$ from *E. coli* (1.3 nmol/A_{260} unit, from Sigma) with [^{14}C]phenylalanine (508 Ci/mmol, from Amersham) and preparation of *N*-acetyl-Phe-tRNA were done as reported (Chinali & Parmeggiani, 1982; Haenni & Chapeville, 1966). Acylation of $tRNA^{Phe}$ was 94 ± 3% for Phe-tRNA and 88 ± 3% for Ac-Phe-tRNA (which was 99.7% free from contamination by Phe-tRNA). Ribosomes were of the "tight couple", about 65% of them bound tRNA (16 ± 0.5 pmol/A_{260} unit), and 90 ± 2% of tRNA-binding ribosomes

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* Address correspondence to this author.

‡ Università di Napoli.

§ University of Louvain Medical School.

[1] Abbreviations: VM and VS, virginiamycin components M and S; EF-G, EF-Tu, and EF-Ts, elongation factors G, Tu, and Ts; EF-T, 1:1 complex of EF-Tu and EF-Ts; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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displayed peptidyl transferase activity.

Ribosomal Complexes Carrying tRNA Derivatives at the P Site. Reaction mixtures for preparation of these complexes contained per milliliter of standard buffer (25 mM Tris-HCl, pH 7.8, 80 mM NH_4Cl, 1 mM dithiotreitol, 7–8 mM $MgCl_2$) 25–50 A_{260} units of ribosomes, 0.1–0.25 mg of poly(U), and $tRNA^{Phe}$ (30–40 pmol/A_{260} unit of ribosomes). Ribosomes were first incubated with poly(U) for 5 min at 30 °C and then with tRNA for 15 min at 30 °C. Binding of [^{14}C]Phe-$tRNA^{Phe}$ or Ac-[^{14}C]Phe-$tRNA^{Phe}$ to the P site was carried out under similar conditions but with a lower concentration of tRNA, 12–17 pmol/A_{260} unit of ribosomes, to minimize A site binding. The reaction mixture for enzymatic binding of Ac-Phe-tRNA contained 0.5 mM GTP, IF-1, -2, and -3, and 5 mM $MgCl_2$. Ribosomal complexes were purified by gel filtration on Sepharose 4B (Chinali et al., 1981).

Ribosomal Complexes Carrying tRNA Derivatives at the A Site. The first step for the preparation of these complexes was as above and resulted in the occupation of the P site by either $tRNA^{Phe}$, Phe-tRNA, or Ac-Phe-$tRNA^{Phe}$ (24–30 pmol of acylated $tRNAs^{Phe}$/A_{260} unit of ribosomes was used in order to fill the site). Nonenzymatic binding of either $tRNA^{Phe}$, Phe-$tRNA^{Phe}$, or Ac-Phe-$tRNA^{Phe}$, 20–50 pmol/A_{260} unit of ribosomes, to the A site was obtained by raising the $MgCl_2$ concentration to 14–15 for 15 min at 30 °C. For the enzymatic binding of Phe-tRNA to the A site, ribosomes were incubated with the Phe-tRNA·EF-Tu·GTP complex at either 7 or 14 mM $MgCl_2$. This ternary complex was prepared by incubating Phe-tRNA, 0.1 mM GTP, and EF-Tu and EF-Ts, 2 and 0.5 mol/mol of Phe-tRNA, for 15 min at 0 °C. Enzymatic binding of Phe-tRNA to the A site of ribosomal complexes carrying Phe-tRNA or Ac-Phe-tRNA at the P site resulted in the formation of Phe-Phe-tRNA or Ac-Phe-Phe-tRNA positioned at the A site and unacylated $tRNA^{Phe}$ at the P site, subsequent to a peptidyl transfer reaction. Complexes were isolated by gel filtration on Sepharose 4B (Chinali et al., 1981).

Translocation of Acylated tRNA. Isolated ribosomal complexes carrying unacylated $tRNA^{Phe}$ at the P site and acylated $tRNA^{Phe}$ at the A site were incubated 5 min at 30 °C with 0.5 mM GTP and EF-G, 20–30 pmol/A_{260} unit of ribosomes, in the presence of either 7 mM or 9.5 mM $MgCl_2$. Under these conditions, the translocation reaction peak was reached within 1 min, as estimated by kinetic analysis with anti-EF-G antiserum.

Analysis of the Composition of tRNA–Ribosome Complexes. Location of Ac-Phe-tRNA at the P or A site was established by incubating isolated ribosomal complexes with 1 mM puromycin before or after preincubation for 5 min at 30 °C with EF-G and GTP. The ratio between the (acetylphenylalanyl)puromycin formed under the two conditions represented the fraction of complexes carrying Ac-Phe-tRNA at the P site. Formation of Phe-Phe-tRNA and Ac-Phe-Phe-tRNA was monitored by hydrolysis of the ribosomal complexes and chromatographic analysis of the resulting dipeptides (Lucas-Lenard & Lipmann, 1967). The amount of Phe-Phe-tRNA present in ribosomal complexes was taken as a measure of occupation of both A and P sites by Phe-tRNA.

Treatment of Ribosome with Virginiamycin M. In most experiments, ribosomal complexes in 40 μL of standard buffer were incubated with either 2.5 or 25 μM virginiamycin M for 3 min at 30 °C prior to addition of a reaction mixture for poly(phenylalanine) synthesis. Isolation of ribosomal complexes by gel filtration did not affect their inactivation by VM. The values of VM concentration that are reported under

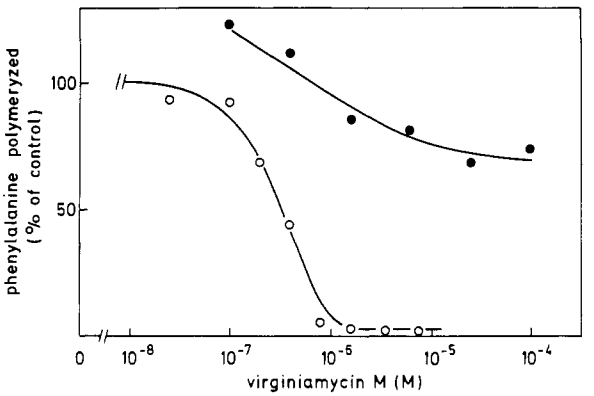

FIGURE 1: Inhibition of poly(U)-directed poly(phenylalanine) synthesis by virginiamycin M as a function of the time of addition. The reaction mixture contained 0.7 A_{260} unit of 70S ribosomes, 5 μg of poly(U), 0.8 mM ATP, 0.35 mM GTP, 50 pmol of $tRNA^{Phe}$, 160 pmol of [^{14}C]phenylalanine, 15 pmol of EF-T, 24 pmol of EF-Tu, 2.5 pmol of EF-G, and 20 milliunits of phenylalanyl-tRNA synthetase in 100 μL of standard buffer with 7 mM $MgCl_2$. Virginiamycin M (2 nmol) was added either 3 min before (○) or 3 min after (●) the onset of the polymerization reaction. Values are expressed as percent of the incorporation observed in the control without antibiotic after 10 (○) or 13 min (●) incubation at 30 °C. In the latter case, the incorporation of phenylalanine after 3 min of incubation, i.e., before VM addition, was subtracted. Controls incorporated 65.8 and 63.9 pmol of phenylalanine, respectively.

Results always represent those present in the final reaction mixture for poly(phenylalanine) synthesis.

Poly(phenylalanine) Synthesis. Reaction mixtures containing EF-Tu, EF-Ts, EF-G, phenylalanyl-tRNA synthetase, $tRNA^{Phe}$, ATP, GTP, and [^{14}C]phenylalanine were preincubated for 10 min at 30 °C to allow aminoacylation of $tRNA^{Phe}$ and formation of EF-Tu·GTP·Phe-tRNA complexes. To 35 μL of these mixtures were added ribosomal complexes, 0.4–0.8 A_{260} units in 40 μL, previously incubated in the presence or absence of VM: after 10 min at 30 °C, radioactivity incorporated into peptides was measured (Chinali et al., 1981). Experimental conditions were chosen so as to obtain a linear incorporation of 60–150 pmol of amino acid/A_{260} unit of ribosomes in the absence of virginiamycin. Protection afforded by bound tRNA derivatives was expressed as percentage of the control activity (absence of the inhibitor). Results were corrected for residual activity of unprotected tRNA-free ribosomes (1–4% of the control) obtained from parallel evaluation.

RESULTS

Definition of the Experimental System. Virginiamycin M permanently altered tRNA-free ribosomes in vitro by inducing a stable conformational change of both acceptor and donor sites of peptidyl transferase (Parfait & Cocito, 1980; Moureau et al., 1983a,b; Di Giambattista et al., 1984; Chinali et al., 1981, 1984). Native polysomes, however, were insensitive to the antibiotic (Contreras & Vazquez, 1977). To test the possibility that peptidyl-tRNA shields ribosomes against an inactivation by VM, a suitable model system has to be developed.

In the experiment illustrated in Figure 1, VM was added to ribosomes either 3 min before or 3 min after the onset of in vitro poly(U)-directed poly(phenylalanine) synthesis. In the first case, maximum inhibition, 96–98%, was already obtained with 10^{-6} M VM, whereas in the latter case a considerable level of synthesis, 75–80% of the control, was observed in the presence of VM concentrations as high as 10^{-4} M. The synthesis of poly(phenylalanyl)[^{3}H]puromycin by isolated ribosome–poly(U)–poly(Phe)-tRNA complexes were also in-

sensitive to VM (not shown). Both observations indicate that poly(U)-directed poly(phenylalanine) synthesis is an appropriate in vitro model system to explore the structural requirements for ribosome protection by tRNA against a VM-promoted inactivation.

To explore these requirements, poly(U)–ribosome complexes carrying various $tRNA^{Phe}$ derivatives at the P site and/or at the A site were incubated with VM for a fixed period of time and then assayed for their residual activity in polypeptide synthesis. In our test, the 100% protection level corresponded to the activity of ribosomal complexes in the absence of VM. Polypeptide synthesis was carried out for 10 min at 30 °C, under conditions in which the polymerization reaction was linear with respect to both time and ribosome concentration, and resulted in the incorporation of 15 ± 3 mol of phenylalanine/mol of active ribosomes in the absence of VM. Experimental conditions were chosen so as to avoid ribosomes running out of mRNA. The 0% level of protection was the phenylalanine incorporation value of tRNA-free poly(U)–ribosome complexes in the presence of VM. We found 96–99% of these complexes to be inactivated by VM. A residual 1–4% activity was observed with all VM-treated ribosome preparations, irrespective of the purification procedure. Analysis of VM-treated ribosomes, which were isolated under conditions of maximal inhibition of poly(phenylalanine) synthesis, pointed to an extremely small fraction of VM-resistant ribosomes, less than 3% of the total, which were responsible for all the residual synthesis of poly(phenylalanine). The remaining ribosomes were found to carry Phe-tRNA and to be inactive in polypeptide chain elongation.

On the basis of preliminary experiments, each ribosomal complex was tested against two VM concentrations, 1.3 and 13 μM, at the fixed incubation time of 3 min at 30 °C. Although the lower VM concentration invariably caused a maximal inhibition, at least 96%, of unshielded ribosomes, the higher concentration of the inhibitor helped to evaluate the level of protection afforded by tRNA derivatives (cf. Figure 1). It should be pointed out that the residual activities of tRNA–ribosome complexes, which were recorded under our experimental conditions, reflect rates rather than yields of VM-induced inactivation, since the percentage of residual active complexes was dependent on the time of exposure to VM (interval tested 1–5 min).

The composition and nature of the complexes used in every experiment was carefully examined, despite the fact that experimental conditions for obtaining each kind of complex were well established. In fact, results could be altered by the presence of small amounts of contaminating complexes displaying a much higher stability toward VM than the main complex tested.

Lack of Ribosome Protection by tRNA Derivatives Bound Directly to the P Site. The mechanism of tRNA-mediated protection of ribosomes against VM was first analyzed by testing the shielding ability of $tRNA^{Phe}$, Phe-$tRNA^{Phe}$, and Ac-Phe-$tRNA^{Phe}$, which were bound nonenzymatically to the P site. As shown in Table I, unacylated $tRNA^{Phe}$ afforded no protection against VM-mediated ribosome inactivation, as judged by the level of poly(phenylalanine) synthesis. This was confirmed under a variety of experimental conditions, i.e., by using different ribosome preparations, varying tRNA/ribosomes ratios, and by exposing ribosome complexes to VM either before or after gel filtration on Sepharose 4B. Phe-$tRNA^{Phe}$ and Ac-Phe-$tRNA^{Phe}$ bound nonenzymatically to the P site had little protective effect. From the data in Table I, it can be estimated that these tRNAs caused at most a 2% protection of ribosomes at the lower VM concentration tested. The slightly higher protection observed in some instances was due to the occupation of the A site either by Phe-Phe-tRNA (occupation of both A and P sites by Phe-tRNA and ensuing peptidization) or by Ac-Phe-tRNA in a small fraction of these ribosomal complexes (see below).

Table I: Ribosome Protection by tRNA Derivatives Directly Bound to the P Site[a]

expt	$tRNA^{Phe}$ derivative positioned at the P site	ribosome-bound tRNA (pmol/A_{260} unit)	correct complex (% of total)[b]	% protection of ribosome activity against 1.3 μM VM	% protection of ribosome activity against 13 μM VM
1	tRNA	15.3[c]	nd[d]	0.4	0.2
2	Phe-tRNA	7.6	nd	1.1	0.6
3	Phe-tRNA	8.4	89	4.0	0.4
4	Ac-Phe-tRNA	8.3	nd	1.6	0.3
5	Ac-Phe-tRNA	9.6	92	4.6	2.0
	Ac-Phe-$tRNA^{enz}$	8.6	100	1.0	0.4

[a] Nonenzymatic binding reactions to P site were made in 7 mM $MgCl_2$ buffer (8 mM $MgCl_2$ in experiment 2) and enzymatic (initiation factors directed) binding of Ac-Phe-tRNA (experiment 5, enz) in 5 mM $MgCl_2$. Ribosomal complexes were incubated with VM and assayed for activity in poly(phenylalanine) synthesis either directly (experiments 1, 2, and 4) or after isolation by gel filtration on Sepharose 4 B (experiments 3 and 5). The last columns give the protection values as percent of the polymerizing activity of control ribosomes. [b] Percentage of total complexes carrying at the P site $tRNA^{Phe}$ derivatives as indicated in the second column [the remaining fraction carried either Ac-Phe-tRNA (experiment 5) or Phe-Phe-tRNA (experiment 3) at the A site]. [c] A total of 95% of available P sites (about 16 pmol/A_{260} unit of ribosomes) occupied by $tRNA^{Phe}$, as measured by the inhibition of Phe-tRNA binding to this site. [d] nd, not done.

Ac-Phe-$tRNA^{Phe}$, which was bound to the P site in the presence of initiation factors, had also little shielding ability. Hence, a direct binding of tRNA derivatives to the P site failed to protect ribosome complexes against VM-promoted inactivation, irrespective of the nature of the tRNA derivative and of the presence of initiation factors.

Shielding Ability of tRNA Derivatives Bound to the A Site. Evidence for the inability of unacylated $tRNA^{Phe}$ positioned at the P site to protect ribosomes against VM was provided in the previous section. Next, the shielding activity of $tRNA^{Phe}$ derivatives bound to the A site of poly(U)–ribosomes carrying $tRNA^{Phe}$ at the P site was explored. Unacylated $tRNA^{Phe}$ was found to be ineffective under a variety of experimental conditions that resulted in a 30–65% occupation of the available A sites, about 16 pmol/A_{260} unit of ribosomes, as judged by the activity of the resulting ribosomal complexes in the EF-G-dependent GTPase reaction (Chinali & Parmeggiani, 1982). One of these assays is reported in experiment 1 of Table II. Phe-$tRNA^{Phe}$ that was bound to the A site either nonenzymatically at 14 mM Mg^{2+} or enzymatically at 7 or 14 mM Mg^{2+} afforded a 20% protection of the ribosomes against 1.3 μM VM (Table II, experiments 2 and 3). Consequently, aminoacyl-tRNA afforded about a tenfold higher protection level when positioned at the A site than when bound directly to the P site. Similar conclusions were drawn for Ac-Phe-tRNA bound nonenzymatically to the A site (Table II, experiment 4).

Protective Effect of tRNA Species Translocated to the P Site. tRNA derivatives can be positioned at the P site either by direct binding or by translocation from the A site. Under Lack of Ribosome Protection by tRNA Derivatives Bound Directly to the P Site, the shielding ability of aminoacyl-tRNA directly bound to the P site was explored and found to be negligible. On the contrary, when Phe-tRNA and Ac-Phe-

Table II: Ribosome Protection by tRNA Derivatives Bound to the A Site and Effect of Translocation to the P Site[a]

expt	$tRNA^{Phe}$ derivatives positioned at the P site	$tRNA^{Phe}$ derivatives positioned at the A site	ribosome-bound tRNA (pmol/A_{260} unit)	correct complexes (% of total)[b]	% protection of ribosome activity against 1.3 μM VM	% protection of ribosome activity against 13 μM VM
1	tRNA	tRNA	8.8[c]	nd[d]	0.1	0.0
2	tRNA	Phe-tRNA	11.9	99	23.2	6.7
	Phe-tRNA	←	11.9	99	18.1	5.2
	tRNA	$Phe\text{-}tRNA^{enz}$	17.0	95	24.8	5.7
	$Phe\text{-}tRNA^{enz}$	←	17.0	95	24.5	6.0
3	tRNA	$Phe\text{-}tRNA^{enz}$	16.4	89	20.1	6.4
	$Phe\text{-}tRNA^{enz}$	←	16.4	89	26.0	10.6
4	tRNA	Ac-Phe-tRNA	8.9	98	14.8	nd
	Ac-Phe-tRNA	←	8.9	98	8.8	nd

[a] Nonenzymatic binding to the A site of poly(U)–ribosome complexes prefilled at the P site with $tRNA^{Phe}$ was carried out at 14 mM $MgCl_2$. Enzymatic (EF-Tu-directed) binding of Phe-tRNA was carried out at 14 (experiment 2) or 7 mM $MgCl_2$ (experiment 3). Translocation from A to P site (EF-G + GTP) (indicated by arrows) was carried out at 9.5 (experiments 2 and 4) or 7 mM $MgCl_2$ (experiment 3). Controls: ribosome–poly(U) complexes carrying $tRNA^{Phe}$ at the P site. [b] Percentage of ribosomal complexes having the composition indicated in the second and third columns [remaining complexes carried either Phe-tRNA at the A site (experiments 2 and 3) or Ac-Phe-tRNA at the P site (experiment 4)]. [c] A total of 55% of available A sites occupied by $tRNA^{Phe}$, as judged by the activity in the EF-G GTPase reaction (Chinali & Parmeggiani, 1982). [d] nd, = not determined.

Table III: Ribosome Protection by Dipeptidyl-tRNA Derivatives Bound to the A Site and Translocated to the P Site[a]

expt	$tRNA^{Phe}$ derivatives positioned at the P site	$tRNA^{Phe}$ derivatives positioned at the A site	ribosome-bound Phe-Phe-tRNA (pmol/A_{260} unit)	% protection of ribosome activity against 1.3 μM VM	% protection of ribosome activity against 13 μM VM
1	tRNA	Phe-tRNA	nd[b]	22.7	2.0
	tRNA	Phe-Phe-tRNA	nd	53.7	20.5
	tRNA	Ac-Phe-Phe-tRNA	nd	58.8	15.0
2	tRNA	Phe-Phe-tRNA	11.2	39.5	13.7
3	tRNA	Phe-Phe-tRNA	14.8	53.2	15.4
	Phe-Phe-tRNA	←	14.8	83.3	25.3
4	tRNA	Ac-Phe-Phe-tRNA	nd	42.5	nd
	Ac-Phe-Phe-tRNA	←	nd	51.5	nd

[a] Nonenzymatic binding of $tRNA^{Phe}$ (experiment 1), $Phe\text{-}tRNA^{Phe}$ (experiments 1–3), and $Ac\text{-}Phe\text{-}tRNA^{Phe}$ (experiments 1 and 4) to the ribosomal P site and subsequent EF-Tu-directed binding of Phe-tRNA to the ribosomal A site were carried out at 7 mM $MgCl_2$. Ribosomal complexes were isolated by gel filtration: after the first binding reaction in experiment 1 and after the second binding reaction in the other experiments. Peptide bond formation between acylated tRNAs present at P and A sites led to formation of dipeptidyl-tRNA. Translocation (EF-G + GTP) from A to P site (indicated by arrows), incubation with VM, and assay of ribosome activity in poly(phenylalanine) synthesis were carried out at 7 mM $MgCl_2$. Controls: ribosome–poly(U) complexes carrying $tRNA^{Phe}$ at the P site. [b] nd = not determined.

tRNA bound to the A site were translocated to the P site by the action of EF-G and GTP, most of their shielding ability was maintained (Table II). Thus, the levels of protection afforded by these $tRNA^{Phe}$ species upon translocation were severalfold higher than those provided by the same tRNAs directly bound to the P site.

The obvious possibility that this result might be secondary to an incomplete translocation was ruled out by performing a kinetic analysis of the translocation reaction. For this purpose, translocation was blocked at different times by addition of an excess of purified anti-EF-G immunoglobulins (Cammarano et al., 1982) and simultaneous temperature shift to 0 °C: the fraction of A sites made free by the reaction was evaluated by measuring the amount of Phe-tRNA binding to ribosomes in the presence of EF-Tu and GTP. According to this test, it was found that in all cases translocation reached its maximum within 1 min at 30 °C, i.e., in a time much shorter than the 5 min used in our experiments. However, while translocation of Ac-Phe-tRNA was virtually complete, only 20–45% of A site bound Phe-tRNA moved to the P site, depending on the Mg^{2+} concentration. The rest of Phe-tRNA–ribosome complexes was found to become inactive in directing poly(phenylalanine) synthesis, suggesting the possibility of an incorrect translocation event. Since in our experiments we measure exclusively the VM-promoted inactivation of the ribosomes capable of polypeptide synthesis, a complete translocation of functional Phe-tRNA–ribosome complexes can be safely assumed to have occurred in every experiment.

Protection Afforded by Dipeptidyl-tRNA at the A Site. Binding of Phe-tRNA to the A site of ribosomes carrying Phe-tRNA or Ac-Phe-tRNA at the P site resulted in peptide bond formation: this led to the occupation of the A site by dipeptidyl-tRNA and of the P site of deacylated $tRNA^{Phe}$. Formation of the first peptide bond markedly increased the level of ribosome protection against VM (Table III, experiment 1). The level of protection approximately correlated with the fraction of ribosomes carrying di-Phe-tRNA (Table III, experiments 2 and 3). Translocation of dipeptidyl-tRNA to the P site resulted in an increase of its shielding ability (20–60% in various experiments). It should be pointed out that, in a few cases, ribosome protection against 1.3 μM VM, which was observed after translocation of dipeptidyl-tRNA to the P site, approached that afforded by polypeptidyl-tRNA [cf. Figure 1 and Chinali et al. (1981)]. The latter tRNA derivative was effective even at high VM concentrations, while di-Phe-tRNA lost most of its shielding action at 13 μM VM. This indicated that ribosomes protected against VM after the first round of elongation did acquire a complete resistance to the antibiotic only upon further elongation of the peptide chain.

In order to compare the results of our experiments, part of which is reported in Tables I–III, the protection value found in each experiment was extrapolated to maximum occupation of the tRNA site by the studied $tRNA^{Phe}$ species under the assumption that protection of ribosomes against VM is linearly related to the fraction of total available A sites or P sites occupied by the tRNA. Table IV reports the mean values of three to four separate experiments. Maximum protection of

Table IV: Maximum Protective Activities of Ribosome-Bound Acylated tRNA^{Phe} Species against Virginiamycin M[a]

ribosomal site occupied by tRNA derivatives	maximum protection of ribosome activity (%) by		
	Phe-tRNA^{Phe}	Ac-Phe-tRNA^{Phe}	Phe-Phe-tRNA^{Phe}
A	25.0 ± 4.6	24.5 ± 3.1	54.2 ± 1.7
P	2.1 ± 0.5	3.5 ● 1.2	
(A→) P	23.6 ± 1.3	17.0 ± 0.4	73.1 ± 6.5

[a] Values represent the percentage of ribosome activity in poly(phenylalanine) synthesis that is expected to be shielded against 1.3 μM VM by a given tRNA^{Phe} species occupying all available A or P sites. Full occupation of A or P site corresponded to 16.4 pmol of either Phe-tRNA or Ac-Phe-tRNA/A_{260} unit of ribosomes and to 14.8 pmol of Phe-Phe-tRNA/A_{260} unit of ribosome (10% of ribosomes had no peptidyl transferase activity). To calculate these values, the percentage of ribosome protection in each experiment was divided by the percentage of ribosomal A or P site occupied by tRNA. The calculation is based on the assumption that there is a linear relationship between ribosome protection against VM-induced inactivation and the fraction of A or P sites occupied by tRNA. Each value represents the mean of three to four experiments. Protection levels afforded by Phe-tRNA at the A site and by Ac-Phe-tRNA at the P site were determined by taking into account complexes formed under both nonenzymatic (two experiments) and enzymatic (two experiments) binding conditions. Protection values in experiments with Phe-tRNA were corrected for the contribution of contaminating Phe-Phe-tRNA and those observed with P site bound Ac-Phe-tRNA for the contribution of Ac-Phe-tRNA possibly present at the A site. Values in the last line refer to protection in the case of P site occupation via translocation from the A site.

ribosome activity was calculated for Phe-tRNA, Ac-Phe-tRNA, and Phe-Phe-tRNA. Phe-tRNA and Ac-Phe-tRNA bound to the A site were about 12 and 7 times more effective than the same species bound directly to the P site. Translocation from the A site to the P site caused a limited reduction of the shielding ability of these tRNAs, and therefore, translocated Phe-tRNA and Ac-Phe-tRNA were about 10 and 5 times more effective, respectively, then the same tRNAs bound directly to the P site. Formation of the first peptide bond increased twofold the protection provided by Phe-tRNA at the A site against 1.3 μM VM. Moreover, translocation of Phe-Phe-tRNA to the P site caused about a 40% increase of the shielding ability of this tRNA.

Discussion

We have previously reported that virginiamycin M causes a stable conformational change of the 50S ribosomal subunits, which entails an inactivation of both the acceptor and the donor sites of peptidyl transferase (Parfait & Cocito, 1980; Moureau et al., 1983a,b; Di Giambattista et al., 1984; Chinali et al., 1981, 1984). As a result, the interaction of aminoacyl-tRNA and peptidyl-tRNA with the ribosomal A and P sites is altered, whereas that of unacylated tRNA remains unaffected (Chinali et al., 1981, 1984). These findings in conjunction with data in the present work suggest that VM specifically alters those parts of the acceptor and donor sites of peptidyltransferase that interact with the aminoacyl and peptidyl moieties of tRNA. Moreover, since the addition of free VM to ribosomes already inactivated by the antibiotic was found to stimulate the release of aminoacyl-tRNA from the ribosomal A site and to inhibit competitively the binding of puromycin to ribosomes (Chinali et al., 1984), it can be inferred that the binding site of VM partly overlaps the peptidyltransferase center on the 50S subunit.

In this paper we have investigated the structural requirements for the protection of ribosomes by tRNA derivatives against inactivation by VM. Our data suggest that the main factor involved is either an aminoacyl or a peptidyl moiety at the 3′-end of tRNA. Accordingly, we propose that the shielding effect of tRNA derivatives relies on the interaction of the acyl moiety of tRNA with peptidyl transferase and is due to a steric hindrance effect between competitors, aminoacyl-tRNA and VM, for overlapping binding sites within the peptidyl transferase domain.

Protection against VM by acylated tRNAs positioned at the A site was found to increase in the order Phe-tRNA < di-Phe-tRNA < poly(Phe)-tRNA. Therefore, the shielding ability of tRNA derivatives may be related to an increase of steric hindrance with the size of their acyl moiety. Moreover, the observation that the stability of ribosomes against pressure-induced dissociation by centrifugation on sucrose density gradients with 3–5 mM Mg^{2+} is increased in the same order by these acylated tRNA^{Phe} derivatives positioned at the A site (Chinali et al., unpublished results) suggests a common ground for the two phenomena. Accordingly, the levels of ribosome protection against inactivation by VM and pressure-induced dissociation should both rely on the increasing strength of interaction of the 3′-end of these tRNA^{Phe} derivatives with the acceptor site of peptidyl transferase. Thus, added aminoacyl residues may increase protection by the peptidyl moiety either by increasing steric hindrance or by stabilizing the association of the 3′-end of tRNA with the 50S subunit.

According to data under Protection Effect of tRNA Species Translocated to the P Site, Phe-tRNA and Ac-Phe-tRNA maintained most of their shielding action against VM when translocated from the A site into the P site by EF-G and GTP. Consequently, these aminoacylated tRNA^{Phe} species afforded a fivefold to tenfold higher protection when they entered the peptidyl site by translocation than by direct binding (see Table IV). Since P site bound substrates do, in fact, behave as active donors in peptidyl-transfer reactions, regardless of the mode of entry, the existence of two kinds of interaction of acylated tRNAs with the donor site of peptidyl transferase ought to be admitted. One type of interaction would be specific for either fMet-tRNA^{fMet} or acylated tRNAs bound directly to the P site, and the other one would be specific for tRNA species translocated by EF-G from the A site. Such a conclusion accounts for previous reports claiming different levels of inhibition afforded by several antibiotics on the formation of the first and of the second peptide bond (Ishitsuka & Kaji, 1972; Watanabe, 1972).

The conventional model of the ribosome assumes that the substrate binding sites of peptidyl transferase are constitutive parts of the tRNA binding sites: the 3′-end of acylated tRNA at the P site interacts with the donor site, and that of tRNA at the A site is bound to the acceptor site of the enzyme. Such a fixed relationship is not consistent with the occurrence of different kinds of interaction of P site bound tRNA species with peptidyl transferase (Table IV). In addition, the conventional model does not account for the action of several peptidyl-transfer inhibitors, hence the earlier proposal by others (Pestka, 1972) of a ribosome model in which each tRNA binding site played alternatively the role of A and P site in subsequent rounds of elongation: such a model, requiring extensive reorientations of ribosomal subunits and tRNAs, is inconsistent with the present knowledge of ribosome structure. We would like to propose here another model whereby translocation occurs without detachment of peptidyl-tRNA and entails an exchange of position, conformation, and function between the two substrate sites of peptidyl transferase. As illustrated schematically in Figure 2A, translocation does require detachment of the 3′-end of peptidyl-tRNA from the acceptor site and its binding to the donor site at each elongation round if, as postulated by the conventional model, these two

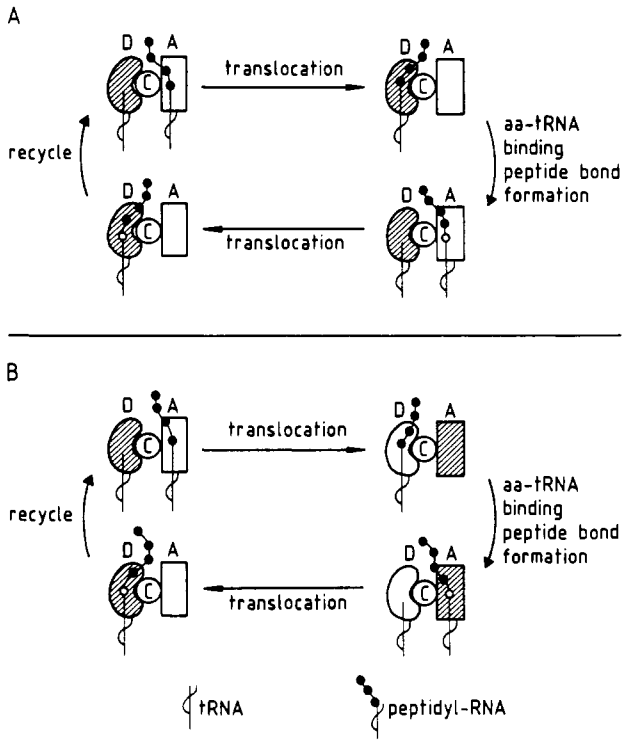

FIGURE 2: Schematic models of the events occurring at the peptidyl transferase center during translocation. Panel A (conventional model): the acceptor (A) and donor (D) sites of the peptidyl transferase have a fixed position with respect to the catalytic center (C). Translocation, thus, always requires the detachment of the 3′-end of peptidyl-tRNA from the acceptor site and its subsequent binding to the donor sites of the enzyme. Panel B (new model): the two sites exchange their position, conformation, and function at each round of translocation. The site receiving peptidyl-tRNA during the peptidization reaction translocates together with its partner. In the figure, the different conformation of acceptor and donor sites is indicated by different shapes, and one of the two substrate sites of peptidyl transferase is cross-hatched to distinguish it from the other site. As can be observed, each site resumes the same position, conformation, and function every two elongation cycles.

sites are fixed and distinct entities. In contrast, our model, illustrated in Figure 2B, postulates that peptidyl-tRNA remains linked to the same substrate site during one elongation cycle and that each peptidyl transferase site does recover the same position, conformation, and function every two elongation cycles. The basic postulates of the conventional model (distinct A and P sites, different functional conformations of acceptor and donor sites of peptidyl transferase, and direct relationship between peptidyl transferase and tRNA sites) are still valid for the new model. The latter, in addition, accounts for previously unexplained observations of virginiamycin M and other peptidyl transferase inhibitors. Indeed, the finding that ribosomes are insensitive to VM during peptide chain elongation and that acylated tRNAs keep most of their shielding action after translocation to the P site suggests that this process might occur without dissociation of peptidyl-tRNA from peptidyl transferase. The constant level of protection of the ester linkage of peptidyl-tRNA against hydrolysis, which is afforded by peptidyl transferase during the whole elongation process, is yet an additional argument in favor of the model we have proposed.

The new model, however, presents a mechanical problem that stems from the positional exchange of peptidyl transferase sites in translocation. A rearrangement of the 3′-end of peptidyl-tRNA ought to be postulated to relieve the torsional stress imposed by the reorientation of the substrate sites of peptidyl transferase during translocation. It has been reported that stable 3′-isomers of acylated tRNA are active both as acceptor and as donor substrates in peptidyl-transfer reaction but are completely unable to translocate (Wagner & Sprinzl, 1983). This suggests that translocation may require a transient transacylation of the peptidyl moiety from the 3′- to the 2′-position of the 3′-terminal ribose of tRNA as expected by our model.

Registry No. Virginiamycin M, 9040-13-5; peptidyl transferase, 9059-29-4.

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