Use of 50 S-binding Antibiotics to Characterize the Ribosomal Site to Which Peptidyl-tRNA Is Bound*

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Five antibiotics (puromycin, erythromycin, lincomycin, sparsomycin, and virginiamycin M1) that bind specifically to the 50 S ribosomal subunit near the peptidyl transferase center were used to compare and characterize the positions of bound AcylPhe-tRNA in the puromycin-reactive and -unreactive states. Binding of the antibiotics was quantitatively measured by their perturbation of fluorescence from probes attached to the ε-amino group of Phe-tRNA. Derivatives of three probes with differing chemical characteristics and environmental sensitivities were used: 3-(4-isothiocyanatophenyl)-7-diethylamino-1-sulfonic acid; poly(A), poly(adenylic acid); poly(U), poly(uridylic acid); pyrene-SAcPhe-tRNA, Phe-tRNA mercaptoacetylated at its ε-amino group; RP-HPLC, reversed-phase high-performance liquid chromatography; s4U8, thio-uracil at position 8 of E. coli tRNA\(^{\text{E}}\); EF, elongation factor.

Recently it was observed in our laboratory (Odom et al., 1990) that puromycin can bind to ribosomes carrying AcylPhe-tRNA\(^{\text{E}}\) in the puromycin-unreactive as well as the puromycin-reactive state, the A and P sites, respectively, of the classical two-site model of ribosome structure (Watson, 1964). This result was not expected from the latter model, in which peptidyl-tRNA is in the P site immediately before peptidyl transfer and in the A site immediately after peptidyl transfer. The result also demonstrates that deacylated tRNA, peptidyl-tRNA, and puromycin can be bound simultaneously to the same ribosome. The finding is consistent with other data, indicating movement of the newly deacylated tRNA, but not the nascent peptide, that is associated with the peptidyl transferase reaction (Hardesty et al., 1986; Odom et al., 1990). Moazed and Noller (1989) reported that nearly all of the same bases on 23 S RNA are protected by AcPhe-tRNA from chemical modification whether it is in the puromycin-reactive or -unreactive state. Considered together these observations appear to indicate that peptidyl-tRNA is in a similar site or position on the 50 S subunit both before and after the peptidyl transfer reaction has taken place, i.e., with or without deacylated tRNA also bound to the ribosome. They prompt the question of why peptidyl transfer to puromycin is blocked when deacylated tRNA is bound to the ribosome. To explore this problem further we have covalently attached fluorescent probes to the ε-amino group of Phe-tRNA by way of a mercaptoacetyl bridge, creating fluorescent peptidyl-tRNA analogues (Odom et al., 1990). These AcylPhe-tRNA derivatives function as analogues of peptidyl-tRNA. Binding of certain 50 S subunit-specific antibiotics causes significant perturbations in fluorescence from the ribosome-bound AcylPhe-tRNAs. The system provides a sensitive way to monitor the environment of the peptidyl-tRNA on the 50 S subunit in the puromycin-reactive and -unreactive sites. Here we report the effects of five antibiotics on fluorescence from three ribosome-bound fluorescent AcylPhe-tRNAs as a method of comparing the puromycin-reactive and -unreactive states. The effects of lincomycin, sparsomycin, and virginiamycin M1, in addition to puromycin and erythromycin, were tested with pyrene, CPM, and AEDANS as fluorescent acyl derivatives. Each of the former three antibiotics is known to be an inhibitor of peptidyl transfer and to bind to the 50 S subunit (for reviews see Ottenheijm et al., 1986; Di-Giambattista et al., 1989). Binding of lincomycin and erythromycin is mutually exclusive (Fernandez-Munoz et al., 1971). They are thought to bind at or near loop V of 23 S RNA, since dimethylation of adenine at position 2058 confers resistance to both antibiotics (Skinner et al., 1983). However, in contrast to lincomycin, erythromycin usually causes no inhibition of peptidyl transfer at least with short peptidyl chains (Cundliffe, 1986). Sparsomycin has been reported to

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be a competitive inhibitor of puromycin binding (Goldberg and Mitsugi, 1967; Pestka, 1972). Virginiamycin M₃, a streptogramin A antibiotic, has been suggested to cause a ribosomal conformational change (DiGiambattista et al., 1987) and to prevent enzymatic binding of aminocycl-TRNA (Cocito and Kaji, 1971).

EXPERIMENTAL PROCEDURES

Materials and Chemicals — Escherichia coli K12, strain A19, was a kind gift from Drs. K. Nierhaus and H. G. Wittmann, Berlin. The colimycin derivatives DCIA, CPM, and CITC were from Molecular Probes, Inc. (Eugene, OR). N-(1-Pyrene)maleimide was purchased from Böhringer, Mannheim, W. Germany. DANS, erythrosine, puromycin dihydrochloride, virginiamycin M₃, lincomycin, and yeast tRNAₕₓ were obtained from Sigma. E. coli tRNAₜₐₚ was from Sub-riden RNA, Inc. (Rolling Bay, WA). [14C]Phenylalanine (420 Ci/mol) was from ICN Life Sciences (Irvine, CA). Phenol and dimethylformamide were from J. T. Baker Chemical Co. and were redistilled before use. All other chemicals were of reagent grade.

Preparation of E. coli Ribosomal Subunits — The growth and maintenance of E. coli K12, strain A19, and the isolation of ribosomes and ribosomal subunits have been described previously (Odum et al., 1980).

Labeling of tRNAₜₐₚ with Fluorescent Probes — E. coli tRNAₜₐₚ was labeled with DCIA at the ε'-U position and purified by RP-HPLC as previously described (Odum and Hardesty, 1987). The labeling and purification of yeast tRNAₜₐₚ at the 5' -phosphate group with a colimycin derivative and of yeast Phe-tRNA at the α-amino group with CPM and IAEDANS has also been described (Odum et al., 1990). Labeling of the α-amino group with N-(1-pyrene)maleimide followed a procedure similar to that for CPM labeling except that the probe was added from a 30 mM stock solution in dimethylformamide. The pyrene-SacPhe-tRNA was purified by RP-HPLC on a Beckman Ultrapure C3 column (0.46 x 7.5 cm) using the elution system described previously (Odum et al., 1988, 1990). Pyrene-SacPhe-tRNA eluted at approximately 45% methanol. The CPM-SacPhe-tRNA was also purified on the C3 column in an improvement over the older phenyl-Sepharose method (Odum et al., 1990). It eluted around 43% methanol.

Aminoacylation and Acetylation of tRNAₜₐₚ — The aminoacylation and acetylation of both yeast and E. coli tRNAₜₐₚ have been described previously (Odum et al., 1990).

Binding of Labeled AcylPhe-tRNA to the Ribosomal Puromycin-reactive or -unreactive Site — Binding to the ribosomal puromycin-reactive site of labeled AcylPhe-tRNA was essentially as previously described (Odum et al., 1989). In a total volume of 0.5 ml, the reaction mixtures contained 50 mM Tris-HCl, pH 7.15, 15 mM Mg(OAc)₂, 100 mM NH₄Cl, 5 mM β-mercaptoethanol, 100 μg of poly(U), 0.01-0.04 A₂₆₀ units of AcylPhe-tRNA, 4.0 A₂₆₀ units of 30 S ribosomal subunits, and 7.2 A₂₆₀ units of 50 S ribosomal subunits. Ribosomes were preincubated with poly(U) for 10 min at 37°C before addition of tRNA and then incubated an additional 20 min at 37°C. When labeled deacylated tRNA was used it was bound to ribosomes by this same procedure. Binding to the puromycin-unreactive site was as described for the puromycin-reactive site except that 0.20 A₂₆₀ units of E. coli tRNAₜₐₚ was present during the preincubation with poly(U).

Puromycin Reaction of Ribosome-bound AcylPhe-tRNA s — For measuring the reactivity of bound AcylPhe-tRNAs with puromycin the reaction was performed in a volume of 0.5 ml at 0°C unless otherwise indicated. For measuring the rate of reaction the reaction time was 10 min; this was increased to 2 h to measure the extent of reaction. The puromycin concentration varied as indicated. The reaction was terminated by adding a 1/9 volume of 2 M NaOAc, pH 5.0. [14C]AcylPhe-puromycin was extracted into 1 ml of ethyl acetate except for AEDANS-labeled material where isobutyl alcohol was used. After vortexing and centrifuging at low speed to separate phases, 0.7 ml of the organic phase was taken for liquid scintillation counting. With fluorescently labeled samples an indication of the puromycin reactivity could also be obtained from fluorescence measurements since after reaction the AcylPhe-puromycin is released from the ribosome, causing a decrease in fluorescence anisotropy.

Binding of Antibiotics to the Ribosome — Stock solutions of the antibiotics were made in water except for virginiamycin M₃ where dimethyl sulfoxide was used and viridaminycin where 50% dimethylformamide was used. When ribosomes were preincubated with antibiotics before binding of AcylPhe-tRNA, the incubation was for 5 min at 37°C with the indicated concentration of antibiotic unless otherwise stated. Frequently fluorescence measurements were performed without antibiotic and then retaken after a 5 min incubation at 37°C with the antibiotic in the fluorescence cuvette.

Fluorescence Measurements — A photon-counting spectrophotometer, Model 8000 from SLM-Aminco, Inc. (Urbana, IL) was used to make steady-state fluorescence measurements. When spectra were taken, data were accumulated at 1-nm intervals with a scanning rate of 0.5 s per wavelength increment. Spectra were automatically corrected for the wavelength dependence of photomultiplier sensitivity. Unless otherwise stated, the sample volume was 0.5 ml and the temperature was 20°C, controlled by a circulating water bath. Fluorescence anisotropy measurements and calculations were made as previously described (Odum et al., 1984). The use of fluorescence anisotropy to determine extent of binding of labeled ligands to the ribosome has also been previously described (Odum et al., 1990).

Calculation of Dissociation Constants — Kₐ values for the various antibiotics with ribosomes carrying tRNA were calculated either from their perturbation of the fluorescence of the labeled deacylated or AcylPhe-tRNAs, from their inhibition of the puromycin reaction, or, in the case of puromycin itself, from the effect of puromycin concentration on the rate of the Michaelis-Menten equation for the velocity of an enzyme-catalyzed reaction. If the rate of the puromycin reaction is significant compared to the rate of dissociation of puromycin from the unreacted ribosome complex, then Kₜ is in the above equation would not be the true dissociation constant but would be analogous to the Kₑ of the Michaelis-Menten equation. The x intercept of a double-reciprocal plot of this equation gives 1/Kₑ. This was the method used to obtain Kₑ values from fluorescence perturbation data.

In the case of the puromycin reaction, if the rate of reaction is slow compared to the binding equilibrium for puromycin, then the following equation holds, $\Delta F = \Delta F_{\text{max}} [A] + K_\text{A} + [P]$, where $\Delta F$ is the observed change in fluorescence intensity, $\Delta F_{\text{max}}$ is the maximum change in fluorescence, obtained when the ribosome is saturated with antibiotic, $[A]$ is the concentration of the antibiotic, and $K_\text{A}$ is the dissociation constant for the antibiotic. The x intercept of a double-reciprocal plot of this equation gives 1/Kₑ. This was the method used to obtain Kₑ values from fluorescence perturbation data. In the case of the puromycin reaction, if the rate of reaction is slow compared to the binding equilibrium for puromycin, then the following equation holds, $v = \frac{V[P]}{K_P + [P]}$, where v is the rate of the reaction at a given puromycin concentration [P], K_P is the dissociation constant for puromycin, and V is the rate of the reaction at a saturating puromycin concentration. This equation is analogous to the Michaelis-Menten equation for the velocity of an enzyme-catalyzed reaction. If the rate of the puromycin reaction is significant compared to the rate of dissociation of puromycin from the unreacted ribosome complex, then Kₑ in the above equation would not be the true dissociation constant but would be analogous to the Kₑ of the Michaelis-Menten equation. The x intercept of a double-reciprocal plot of this equation gives 1/Kₑ. This was the method used to obtain Kₑ values from fluorescence perturbation data. If the assumption of a steady state is not strictly valid for this situation, since the ribosome complex is not regenerated upon reaction with puromycin. Thus an approximation of linearity is obtained only under conditions where the extent of the reaction is low.

A double-reciprocal plot according to this equation has an x intercept of 1/Kₑ. This plot was used to obtain Kₑ values for puromycin from kinetic data. The averages and standard deviations of three kinetic determinations of the Kₑ values for puromycin are 109 ± 54 μM with unlabeled AcPhe-tRNA, 48 ± 15 μM with CPM-SacPhe-tRNA, and 21 ± 1 μM with pyrene-SacPhe-tRNA.

RESULTS

Puromycin — As reported earlier (Odum et al., 1990), puromycin binds to ribosomes bearing deacylated tRNA and AE-DANS-SacPhe-tRNA or CPM-SacPhe-tRNA in the puromycin-unreactive state or functional ribosomal site, causing large changes in fluorescence parameters. With pyrene-

SacPhe-tRNA in the puromycin-unreactive site, we find that

puromycin causes about a 60% increase in fluorescence intensity and a slight red shift. Using this change in fluorescence intensity to obtain a saturation curve for puromycin, we estimate the Kₑ of the pyrene derivative to be 0.5 mM at 20°C. Using similar changes in fluorescence to monitor binding we estimated the dissociation constant for puromycin to be in the range of 0.5 mM at 20°C with either AEDANS-SacPhe-tRNA or CPM-SacPhe-tRNA as the fluorophore.
Values for $K_d$ at 0 °C, the temperature at which the puromycin reaction is usually carried out, are somewhat lower (Table I).

It is difficult to measure binding of puromycin in the puromycin-reactive site. After binding, peptidyl transfer to puromycin occurs rapidly with subsequent release of peptidylpuromycin from the ribosome. By measuring fluorescence at low temperature and immediately after the addition of puromycin, however, it is possible to separate binding from the covalent reaction, with CPM-SAcPhe-tRNA and pyrene-SAcPhe-tRNA. In the case of AEDANS-SAcPhe-tRNA the peptidyl transferase reaction is so rapid even at 0 °C that it has not been possible to separate these events. With CPM-SAcPhe-tRNA, puromycin binding affects fluorescence in a similar manner when the tRNA is in either the puromycin-reactive or -unreactive site, giving a 7-nm blue shift and slight increase in intensity as reported previously (Odom et al., 1990). Utilizing this shift, we estimate $K_d$ for puromycin binding to ribosomes carrying CPM-SAcPhe-tRNA in the puromycin-reactive site to be about 60 μM at 0 °C, about 5-fold lower than for ribosomes carrying CPM-SAcPhe-tRNA in the puromycin-unreactive site. With pyrene-SAcPhe-tRNA in the puromycin-reactive site puromycin binding gives little direct effect on the fluorescence so that a $K_d$ value cannot be determined fluorescently. It is also possible to estimate dissociation constants for puromycin by measuring the effect of puromycin concentration on the rate of peptidyl transfer to puromycin (see, for example, Pestka, 1972). By performing the puromycin reaction at 0 °C for 10 min, satisfactory saturation curves for puromycin are obtained using AcPhe-tRNA, CPM-SAcPhe-tRNA, or pyrene-SAcPhe-tRNA. Under these conditions less than 30% of the peptidyl-tRNA analogues have reacted at satuating puromycin concentrations. Values for $K_d$ calculated by this method are shown in Table I. The value obtained with CPM-SAcPhe-tRNA (48 μM) is similar to the 60 μM obtained by fluorescence. With AEDANS-SAcPhe-tRNA the rate of the peptidyl transferase reaction with puromycin is too high for binding to be measured reliably, as indicated above. There is considerable variation in $K_d$ depending on the type of AcylPhe-tRNA used, nonfluorescent AcPhe-tRNA giving the highest value (109 μM) and pyrene-SAcPhe-tRNA giving the lowest value (21 μM). The range of $K_d$ values obtained suggests that the affinity for puromycin is dependent on the nature of the peptidyl moiety. This is consistent with the finding of Pestka (1972) that polysomes show much higher affinity for puromycin than ribosomes carrying only AcPhe-tRNA.

**Erythromycin**—Erythromycin was shown previously to affect the fluorescence of ribosomes-bound CPM-SAcPhe-tRNA and AEDANS-SAcPhe-tRNA (Odom et al., 1991). The effects were similar whether the tRNAs were bound to the puromycin-reactive or -unreactive site. Using pyrene-SAcPhe-tRNA we found a somewhat larger disparity in the magnitude of the fluorescence perturbation produced by erythromycin in the two states, a 180% increase in fluorescence intensity being produced in the puromycin-reactive state (Fig. 1) in contrast to only a 30% increase in the puromycin-unreactive state (Table II). Without erythromycin the fluorescence quantum yield of puromycin-unreactive pyrene-SAcPhe-tRNA is about 25% higher than that of puromycin-reactive material. The affinity for erythromycin of ribosomes bearing any of the labeled AcylPhe-tRNAs in either the puromycin-reactive or -unreactive site is relatively high. In fact, the antibiotic appears to bind more strongly to such ribosomes than to empty ribosomes, since substoichiometric concentrations of erythromycin give disproportionately large effects on fluorescence. For example, 0.1 μM erythromycin gave more than half of the maximal erythromycin effect when added to 0.6 μM ribosomes containing 25 nM CPM-SAcPhe-tRNA (0.25 times the concentration of erythromycin) in either the puromycin-reactive or -unreactive site (data not shown). This result indicates that erythromycin binds preferentially to the ribosomes to which CPM-SAcPhe-tRNA is bound. Thus, although erythromycin cannot bind to ribosomes containing nascent peptides longer than a few amino acid residues (Tai et al., 1974; Odom et al., 1991), the fluorescent peptidyl-tRNA analogues used in this study enhance erythromycin binding suggesting that the size and character of the peptidyl moiety may be critically important. Interestingly, the reaction with puromycin of CPM-SAcPhe-tRNA, but not that of the other

![Fig. 1. The effect of various antibiotics on the fluorescence of pyrene-SAcPhe-tRNA in the puromycin-reactive state. Samples contained 300-pmol ribosomes and 30-pmol pyrene-SAcPhe-tRNA in a volume of 500 μL. ——, no additions; ——, +2 mM lincomycin; ——, +2 μM erythromycin; ——, +20 μM sparsomycin; ——, ribosome blank.](image-url)
TABLE III
Effect of lincomycin on the fluorescence of labeled AcylPhe-tRNAs in the puromycin-reactive and -unreactive ribosomal sites, with calculated dissociation constants of the antibiotic

| Label on AcylPhe-tRNA/site | ΔI* | Δλmax* | Lincomycin Kd |
|---------------------------|-----|--------|---------------|
| Pyrene/puromycin-reactive | +195 | 0 | 300 μM |
| Pyrene/puromycin-unreactive | +30 | 0 | 950 μM |
| CPM/puromycin-reactive | +3 | +2 | 30 μM |
| CPM/puromycin-unreactive | 0 | -3 | 1100 μM |
| AEDANS/puromycin-reactive | -34 | 0 | 2 μM |
| AEDANS/puromycin-unreactive | -29 | 0 | 1200 μM |

*ΔI and Δλmax are the changes in fluorescence intensity and emission maximum, respectively, observed after the addition of 5 mM lincomycin. Measurements were made at 20 °C.

labeled AcylPhe-tRNAs, greatly inhibited by erythromycin. Lincomycin—Changes in fluorescence caused by binding of lincomycin to ribosomes carrying each of the three fluorescently labeled AcylPhe-tRNAs in either the puromycin-reactive or -unreactive ribosomal site are shown in Table III. The largest change is observed using pyrene-SAceph-tRNA, which in the puromycin-reactive site undergoes a 195% increase in fluorescence upon addition of lincomycin (Fig. 1). In the puromycin-unreactive site the increase in fluorescence is only 30%. These changes are similar to those given by erythromycin, as considered above. They are consistent with the conclusion that the two antibiotics have overlapping binding sites. Lincomycin, like erythromycin, quenches the fluorescence of AEDANS-SAceph-tRNA to about the same extent in either ribosomal site. However, lincomycin, in contrast to erythromycin, shifts the emission maximum of CPM-SAceph-tRNA in opposite directions in the two ribosomal sites, causing a red shift in the puromycin-reactive site and a blue shift in the puromycin-unreactive site. The perturbation of fluorescence observed upon binding of lincomycin was used to obtain saturation curves from which dissociation constants were calculated (Table III). Large differences in the dissociation constant are observed depending on which of the fluorescently labeled AcylPhe-tRNAs is bound and whether it is in the puromycin-reactive or -unreactive site. That lincomycin binding, like that of erythromycin, is influenced by the nature of the peptidyl moiety is indicated from the previous observation that it does not bind to native polyribosomes unless their nascent peptides are first removed (Contreras and Vazquez, 1977). In the puromycin-reactive site the lowest Kd (approximately 2 μM) for lincomycin is obtained with AEDANS-SAceph-tRNA. By contrast, with ribosomes carrying CPM-SAceph-tRNA or pyrene-SAceph-tRNA in the puromycin-reactive site the Kd values are 20 and 300 μM, respectively. In the puromycin-unreactive site Kd values for lincomycin are near 1 mM with all of the labeled AcylPhe-tRNAs tested. Thus in all cases the affinity of lincomycin for the puromycin-reactive state is greater than that for the puromycin-unreactive state, but the magnitude of the difference varies from about 3-fold for pyrene-labeled tRNA to 600-fold for AEDANS-labeled tRNA.

Sparsomycin—Sparsomycin causes relatively small changes in the fluorescence of all of the labeled AcylPhe-tRNAs in the ribosomal puromycin-reactive site (Table IV and Fig. 1). Kd values were estimated from these changes. However, in general the fluorescence perturbations induced by sparsomycin are less than those produced by the other antibiotics tested. Previous observations (Pestka, 1974) indicated that sparsomycin binding is enhanced rather than inhibited by the presence of nascent peptides, suggesting that the antibiotic does not compete with the nascent peptide for a common binding site. This is also suggested by the fact that the Kd for sparsomycin binding to ribosomes bearing labeled decacylated tRNA is higher than that for ribosomes bearing AcylPhe-tRNA (Table IV). The fluorescence titrations for the puromycin-reactive state indicate very tight binding of sparsomycin with AEDANS-SAceph-tRNA, but somewhat weaker binding with pyrene-SAceph-tRNA and CPM-SAceph-tRNA, the Kd with the latter being about 0.1 μM (Table IV). Sparsomycin had no detectable effect on fluorescence from any of the three AcylPhe-tRNAs bound in the puromycin-unreactive site but did cause quenching and a small blue shift in the fluorescence of AcPhe-tRNA fluorescein labeled in position 8 with a coumarin derivative. This effect required relatively high sparsomycin concentrations (20 μM or higher, data not shown). However, surprisingly, it was found that sparsomycin has a large effect on the fluorescence of AEDANS-SAceph-tRNA in the puromycin-unreactive site in the presence of puromycin. Previously we had shown (Odom et al., 1991) that puromycin alone causes a large increase in the fluorescence of AEDANS-SAceph-tRNA in this site. Sparsomycin causes a decrease and red shift in this fluorescence (Fig. 2). A double-reciprocal plot (not shown) constructed from the sparsomycin saturation curve gave an apparent Kd of 27 μM for sparsomycin. This is about 3 orders of magnitude higher than the corresponding value with AE-

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DANS-SAcPhe-tRNA in the puromycin-reactive site. If one assumes that sparsomycin and puromycin are competitive for binding as previously reported (Goldberg and Mitsugi, 1967; Pestka, 1972) then the true $K_d$ for sparsomycin binding in the presence of puromycin would be the apparent $K_d$ divided by $[1 + (P/K_d)]$ where $P$ is the puromycin concentration and $K_d$ is the dissociation constant for puromycin, previously calculated to be about 0.5 mM at 20 °C (Odum et al., 1990).

Since the puromycin concentration was 2 mM, the true $K_d$ for sparsomycin would be 1/5 of the apparent $K_d$ still much higher than the value obtained for the puromycin-reactive state. However, the same apparent $K_d$ value for sparsomycin was obtained whether the sparsomycin titration was performed in the presence of 0.5 or 2 mM puromycin, indicating little or no competition for binding between sparsomycin and puromycin. We also have evidence, contrary to literature reports cited above, that sparsomycin and puromycin can be bound simultaneously to ribosomes bearing AcylPhe-tRNA in the puromycin-reactive site.$^3$

**Virginiamycin M$_1$**—Binding of virginiamycin M$_1$ to ribosomes also was measured by its effect on the fluorescence from the AcylPhe-tRNA$_{\text{PSAcPhe-tRNA}}$. First the effect of preincubation of ribosomes with virginiamycin M$_1$ on binding of deacylated tRNA$_{\text{PSAcPhe-tRNA}}$, AcylPhe-tRNA, or AcylPhe-tRNA after deacylated tRNA$_{\text{PSAcPhe-tRNA}}$ was determined. The deacylated tRNA$_{\text{PSAcPhe-tRNA}}$ was labeled at either the 5'-phosphate, or at the thioracil at position 8, both with coumarin derivatives. The AcylPhe-tRNA$_{\text{PSAcPhe-tRNA}}$ used were labeled at these positions or at the α-amino group with CPM. Table V indicates that preincubation with virginiamycin M$_1$ has little effect on binding of deacylated tRNA or AcylPhe-tRNA alone, but largely prevents the binding of AcylPhe-tRNA after prebinding of deacylated tRNA. This AcylPhe-tRNA$_{\text{PSAcPhe-tRNA}}$ would be bound into the puromycin-unreactive site. A previous report (Cocito and Kaji, 1971) has indicated that virginiamycin M$_1$ blocks EF-Tu-dependent binding of aminocyl-tRNA to the acceptor site of ribosomes. It also appears to prevent puromycin binding.$^3$ We (Odum et al., 1990) and others (Mozzad and Noller, 1989) have presented evidence that puromycin-unreactive AcylPhe-tRNA$_{\text{PSAcPhe-tRNA}}$ is bound in a site distinct from the unblocked aminocyl-tRNA$_{\text{PSAcPhe-tRNA}}$ binding site, presumably the site for puromycin binding. In any event, the present results clearly indicate that virginiamycin M$_1$ prevents binding of AcylPhe-tRNA$_{\text{PSAcPhe-tRNA}}$ to ribosomes to which deacylated tRNA is prebound. Other experiments with virginiamycin M$_1$ utilized ribosomes bearing prebound fluorescently labeled AcylPhe-tRNA in either the puromycin-reactive or -unreactive site. In the latter situation, however, no effect of virginiamycin M$_1$ could be detected, supporting the conclusion that virginiamycin M$_1$ does not bind to such ribosomes.

Thus binding of virginiamycin M$_1$ to ribosomes and binding of AcylPhe-tRNA$_{\text{PSAcPhe-tRNA}}$ to the puromycin-unreactive site appear to be mutually exclusive. Virginiamycin M$_1$ does bind to ribosomes carrying labeled AcylPhe-tRNA in the puromycin-reactive site, and to ribosomes bearing 5'-phosphate-labeled deacylated tRNA, as indicated by its perturbation of their fluorescence (Table VI), as well as by its inhibition of the reaction of the AcylPhe-tRNA$_{\text{PSAcPhe-tRNA}}$ with puromycin (data not shown). The largest direct effect of virginiamycin M$_1$ on fluorescence occurs with AEDANS-SAcPhe-tRNA, with which a 7-nm red shift and more than a 2-fold increase in fluorescence intensity are observed. With pyrene-SAcPhe-tRNA there is little direct effect of virginiamycin M$_1$ while with CPM-AcylPhe-tRNA there is only a slight red shift in the fluorescence spectrum. Titration with virginiamycin M$_1$ of ribosomes carrying AEDANS-SAcPhe-tRNA indicates a $K_d$ for virginiamycin M$_1$ of about 2 μM, much higher than the value of < 10 nM determined for binding of virginiamycin M$_1$ to empty ribosomes.$^3$ The binding of virginiamycin M$_1$ to ribosomes carrying CPM-SAcPhe-tRNA is even weaker, for which fluorescence titration indicates a $K_d$ near 10 μM. Since there is little direct effect of virginiamycin M$_1$ on fluorescence from bound pyrene-SAcPhe-tRNA, competition between lincomycin and virginiamycin (to be reported in more detail elsewhere) was used to obtain a $K_d$ of 0.5 μM.

**DISCUSSION**

Earlier results (Hardesty et al., 1986; Odum et al., 1990) indicated that peptidyl-tRNA was in a similar physical position on 50 S ribosomes before and after the peptidyl transferase reaction and that puromycin as well as deacylated tRNA could be bound to the ribosomes in the latter situation. Peptidyl transfer to puromycin does not take place in the latter situation. These observations raise the question of how deacylated tRNA blocks the peptidyl transferase reaction and about the nature of the peptidyl-tRNA binding site in the two situations. The effect of the antibiotic binding on fluorescence...
from the ribosomal complex provides an exquisitely sensitive measure of changes in the position and environment of labeled aminoacyl-tRNA in the two functional states. Comparisons between the puromycin-reactive and -unreactive states can be summarized as follows: 1) Puromycin can bind to ribosomes carrying any of the three labeled AcylPhe-tRNAs in the puromycin-unreactive site, but the $K_D$ for puromycin with such ribosomes is 2.0-0.5 mm, as compared with 0.1 mm or less for ribosomes carrying the labeled AcylPhe-tRNA in the puromycin-reactive site. 2) Efthromycin binds tightly to both states of ribosomes using any of the labeled AcylPhe-tRNAs. In contrast to the results with CPM-SAcPhe-tRNA and AEDANS-SAcPhe-tRNA, however, efthromycin has a larger effect on the fluorescence of pyrene-SAcPhe-tRNA in the puromycin-reactive state than in the puromycin-unreactive state. 3) Lincomycin, while binding to ribosomes with AcylPhe-tRNA in either state, binds less strongly to ribosomes with puromycin-unreactive AcylPhe-tRNA. Also, the binding of lincomycin to the puromycin-unreactive state varies less with different labeled AcylPhe-tRNAs than does binding to the puromycin-reactive state. 4) Sparsomycin also can bind to ribosomes with AcylPhe-tRNA in either state, but, like puromycin and lincomycin, binds more tightly to ribosomes with AcylPhe-tRNA in the puromycin-reactive state compared to the corresponding puromycin-unreactive state. 5) Virginiamycin M, apparently cannot bind to ribosomes with labeled AcylPhe-tRNA in the puromycin-unreactive state, in contrast to the case of labeled AcylPhe-tRNA in the puromycin-reactive state. Conversely, ribosomes preincubated with virginiamycin M do not bind AcylPhe-tRNA into the puromycin-unreactive state.

Thus, the present results with each of the antibiotics indicate that there are quantitative differences between the states. Indeed, Moazed and Noller (1989) detected one difference between the two states on the 50 S subunit. Residue Ade-2602 of 23 S RNA was significantly protected by AcPhe-tRNA in the puromycin-unreactive state, while its reactivity was enhanced in the puromycin-reactive state. They proposed a so-called hybrid ribosomal state, in which after peptidyl transfer the incoming tRNA of the new peptidyl-tRNA moves into the P site of the 50 S subunit but remains in the A site of the 30 S subunit, whereas the newly decylated tRNA moves into the exit site of the 50 S subunit but remains in the P site of the 30 S subunit. The EF-G reaction would then move the tRNAs with respect only to the 30 S subunit. It follows that there are three sites to which tRNA can bind on the large subunit but only two on the small subunit. All of the results from our laboratory including those reported here appear to be directly supportive of or at least generally consistent with this model.

Barta et al. (1990), measuring the effect of puromycin on photocross-linking from peptidyl-tRNA to 50 S ribosomal components, also concluded that puromycin must be binding simultaneously with puromycin-unreactive peptidyl-tRNA. However, their interpretation of this binding was different from ours. They concluded that puromycin displaced the 3'-end of the peptidyl-tRNA from its binding site but that the peptidyl-tRNA remained bound to the ribosome due to attachment at other points. In the light of our present finding that the dissociation constant for puromycin is generally higher for the puromycin-unreactive ribosomal state than for the puromycin-reactive state, the hypothesis of Barta et al. (1990) cannot be ruled out completely. We think that several considerations, however, argue against this hypothesis and that the puromycin binding site is distinct from that of peptidyl-tRNA. First, since displacement of the 3’-end of the peptidyl-tRNA by puromycin would involve not only breakage of the bonds at the binding site but also presumably a conformational change in the tRNA in this region, it seems likely that this would cause a larger increase in the $K_D$ for puromycin than is observed. The $K_D$ for the puromycin complex measured with only AcPhe-tRNA bound to the ribosomes (the reactive state, Table I) is 109 $\mu$M, corresponding to a change in free energy of $-5.4$ kcal/mol. This may be a reasonable estimate of the contribution of the amino acid-adenosine sequences at the 3’-end of aminoacyl-tRNA to its binding to 70 S ribosome. Puromycin is a functional analogue of aminoacyl-tRNA and is structurally similar to this 3’-sequence. In contrast, formation of the N-blocked aminoacyl-tRNA-70 S ribosome complex is essentially irreversible under the conditions used; by our estimate $K_D$ is below $10^{-10}$ M corresponding to a $\Delta G$ of more than 13 kcal/mol. However, the difference in $K_D$ for puromycin in the reactive and unreactive state with any of the three AcyPhe-tRNAs is less than 10-fold, corresponding to a difference in free energy of less than 1.4 kcal/mol for the two states. For comparison, the difference in $K_D$ for the puromycin-ribosome complex that is associated with binding pyrene-SAcPhe-tRNA rather than AcPhe-tRNA to the reactive state is about 5-fold (0.7 kcal/mol). Second, puromycin has nearly identical effects on fluorescence parameters of CPM-SAcPhe-tRNA in either the puromycin-reactive or -unreactive states (Odom et al., 1990). Third, the results of Moazed and Noller (1989) using an independent technique, as discussed above, indicate that the pattern of protection given by AcPhe-tRNA in the puromycin-unreactive state is different from that given by Phe-tRNA, the latter presumably corresponding to the classical acceptor site.

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