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# FUNCTIONAL INTERACTION OF NEOMYCIN B AND RELATED ANTIBIOTICS WITH 30S AND 50S RIBOSOMAL SUBUNITS

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Summary. Antibiotics of the neomycin, kanamycin and gentamicin, but not streptomycin, groups stabilize the GDP·elongation factor (EF) G·50S subunit ·fusidic acid complex. Treatment of 30S subunits, but not of 50S subunits, with neomycin B or kanamycin B, followed by removal of excess unbound antibiotic and supplementation with untreated complementary subunits, promotes poly(U)-dependent binding of Tyr-tRNA to the reassociated ribosomes (misreading). A similar treatment of either ribosomal subunit with neomycin B inhibits the EF-G-dependent translocation of Ac-Phe-tRNA. These results suggest that interaction of neomycin B and related antibiotics with the 30S subunit induces misreading and inhibits translocation, and interaction with the 50S subunit stabilizes EF-G on the ribosome and also inhibits translocation.

Introduction. Streptomycin and the closely related antibiotics dihydrostreptomycin and bluensomycin bind specifically to only one ribosomal site located on the 30S ribosomal subunit (1, review). In contrast, the patterns of misreading and polypeptide synthesis inhibition induced by aminoglycoside antibiotics of the neomycin, kanamycin and gentamicin groups (as well as the failure to isolate one step ribosomal mutants highly resistant to these antibiotics) suggest that these drugs interact with multiple ribosomal sites (2-5). In fact, [$^3$H]kanamycin has been shown to bind with high affinity to both 30S and 50S subunits, and neomycin and gentamicin (but not streptomycin) strongly depress binding (6).

Besides interferring with aminoacyl-tRNA recognition, these antibiotics inhibit peptidyl-tRNA translocation (6,7) and stabilize the EF-G plus guanosine nucleotide binding to the ribosome (8). These effects, however, have not yet been correlated with specific binding of the antibiotics to either the 30S or 50S or to both ribosomal subunits. Our aim in this work has been to define such correlations.

Materials and Methods. E. coli MRE600 ribosomal subunits derived from 1 M $NH_4Cl$-washed 70S ribosomes, EF-G, and EF-T (EF-Tu plus EF-Ts) were prepared as described elsewhere (9-11). Purified commercial $tRNA^{Phe}$ and $tRNA^{Tyr}$ (Sigma, USA) were charged with [$^{14}$C]phenylalanine (870 cpm/pmol) and [$^3$H]tyrosine (2100 cpm/pmol) respectively, and the resulting [$^{14}$C]Phe-tRNA was acetylated. Sources of antibiotics have been previously described (7).

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Prior to each experiment 30S subunits were activated by incubation at 40° for 30 min in 200 mM $NH_4Cl$, 20 mM Tris-HCl pH 7.8, 20 mM magnesium acetate, 2 mM dithiothreitol. 50S subunits and 70S ribosomes were activated at 30°for 30 min in 60 mM $NH_4Cl$, 40 mM KCl, 10 mM Tris-HCl pH 7.8, 20 mM magnesium acetate, 1 mM dithiothreitol. Treatment of ribosomal particles with antibiotics was performed in mixtures (95-125 µl) containing either 2.7 µM 30S subunits, 2.3 µM 50S subunits or 1.3 µM 70S ribosomes and antibiotics as specified, in the same ionic environment as the activation mixtures, excepting magnesium acetate which was lowered to 12 mM. After incubation at 30° for 10 min, excess unbound antibiotic was removed by Sepharose 6B filtration at room temperature in a column (0.6 x 15 cm) equilibrated with 65 mM $NH_4Cl$, 20 mM Tris-HCl pH 7.8, 12 mM magnesium acetate and 6 mM 2-mercaptoethanol. Other methods are specified in the legends to Tables and Figure.

Results. *Stabilization of GDP·EF-G·50S subunit·fusidic acid complex.* Our recent work has shown that aminoglycoside antibiotics stabilize the guanosine nucleotide·EF-G·70S ribosome complexes (8). Table I shows that many aminoglycosides also stabilized the [$^3$H]GDP·EF-G·50S-subunit·fusidic acid complex, and as with complexes containing 70S ribosomes (8), neomycin B, gentamicin C1a, kanamycin B and tobramycin were the most active drugs. In

Table I. Effect of aminoglycoside antibiotics (0.1 mM) on the dissociation of [$^3$H]GDP·EF-G·50S-subunit·fusidic acid complexes

| Antibiotic | [$^3$H]GDP released (%) |
|---|---|
| None | 100 |
| Neamine | 84 |
| Ribostamycin | 83 |
| Paromomycin | 74 |
| Neomycin C | 60 |
| Neomycin B | 22 |
| Gentamicin C1 | 83 |
| Gentamicin C1a | 30 |
| Sisomicin | 58 |
| Verdamicin | 48 |
| Kanamycin A | 79 |
| Kanamycin B | 29 |
| Tobramycin | 42 |
| Streptomycin | 113 |
| Dihidrostreptomycin | 105 |
| Bluensomycin | 96 |

[$^3$H]GDP·EF-G·50S-subunit·fusidic acid complexes were formed in mixtures containing: 12 mM $NH_4Cl$, 4 mM KCl, 10 mM Tris-HCl pH 7.8, 10 mM magnesium acetate, 1 mM dithiothreitol, 2.5 mM fusidic acid, 7.5 $A_{260}$ units/ml 50S subunits, 45 µg/ml EF-G and 1 µM [$^3$H]GDP (2050 cpm/pmol). After 30 min at 30°, complexed [$^3$H]GDP (0.73-0.96 molecules/50S subunit) was determined by diluting 10 µl samples with 4 ml of dilution buffer (10 mM $NH_4Cl$, 10 mM Tris-HCl pH 7.8, 10 mM magnesium acetate, 2 mM fusidic acid and 0.1 mg/ml bovine serum albumin) and filtration through nitrocellulose membranes (8,9). Other 10 µl portions were diluted with 4 ml of dilution buffer prewarmed to 30° and containing the specified antibiotics at 0.1 mM. After 2 min of incubation at 30°, the amount of [$^3$H]GDP remaining in complex was determined. Released [$^3$H]GDP was calculated by difference and, in controls without antibiotic, was 0.25-0.44 molecules/50S subunit.

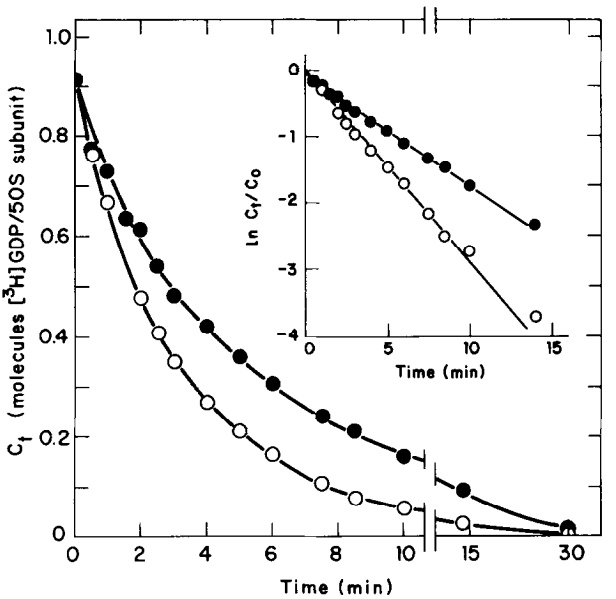


Fig. 1. Effect of 7 μM neomycin B on the decay of [$^3$H]GDP·EF-G·50S-subunit ·fusidic acid complex. Complex was formed in a 0.46 ml reaction mixture as described in the legend to Table I. 0.2 ml samples were diluted into 80 ml of dilution buffer at 30° with (●) or without (○) 7 μM neomycin B, 5 ml portions were removed at time intervals and the amount of [$^3$H]GDP remaining in complex ($C_t$) was determined. The data obtained were replotted in the inset, Co being the amount of [$^3$H]GDP in complex at the start of the decay (0.91 molecules/50S subunit). Straight lines were fitted by the least square method and apparent dissociation constants were calculated from their slopes.

contrast, antibiotics of the streptomycin group (streptomycin, dihidro-streptomycin and bluensomycin), which interact with the 30S subunit (1) and are active on 70S ribosome-containing complexes (8), had no effect. Thus, there is a site(s) on the 50S subunit for the action of neomycin B and related antibiotics.

Figure 1 shows that relatively low concentrations of neomycin B (7 μM) substantially slowed complex dissociation. Moreover, the dissociation with and without antibiotic followed first order kinetics, the apparent rate constants being $2.7 \times 10^{-3}$ $sec^{-1}$ with neomycin B and $4.1 \times 10^{-3}$ $sec^{-1}$ without it (Fig. 1, inset). In similar experiments 0.1 mM sisomicin or kanamycin B decreased the rate constant to $2.3 \times 10^{-3}$ $sec^{-1}$ or $2.0 \times 10^{-3}$ $sec^{-1}$, respectively.

*Induction of misreading.* It has been reported that streptomycin does not induce misreading in isolated 30S subunits (12). Under a great variety of conditions

Table II. Effect of treating ribosomal particles with 2 μM neomycin B on the EF-Tu plus GTP-dependent binding of [$^3$H]Tyr-tRNA to poly(U)-programmed ribosomes

| Ribosomal particles treated before Sepharose filtration | Neomycin B addition during assay | [$^3$H]Tyr-tRNA binding (molecules/ribosome) |
|---|---|---|
| None | - | 0.06 ( 7) |
| None | + | 0.87 (100) |
| 30S | - | 0.57 ( 66) |
| 30S | + | 0.82 ( 94) |
| 50S | - | 0.05 ( 6) |
| 50S | + | 0.72 ( 83) |
| 70S | - | 0.59 ( 68) |
| 70S | + | 0.84 ( 97) |

The ribosomal particles indicated in the Table were treated with 2 μM neomycin B and the excess antibiotic was removed by Sepharose filtration (Methods). Binding of [$^3$H]Tyr-tRNA to ribosomes was performed in mixtures (60 μl) containing: 5 pmol treated particles, 5 pmol complementary untreated particles (except when 70S treated particles were used), 15 μg/ml poly(U), 11 pmol [$^3$H]Tyr-tRNA, 0.1 mM GTP, 20 μg/ml EF-T and, when indicated, 2 μM neomycin B. Ionic conditions were as in the buffer for column elution (Methods). After incubation at 30° for 10 min, bound [$^3$H]Tyr-tRNA was determined by the nitrocellulose filter technique (9). Binding in control mixtures without poly(U) was 0.03-0.06 molecules [$^3$H]Tyr-tRNA per ribosome; these values have been subtracted. Figures in brackets indicate binding as percentages of that occurring in the mixture with untreated particles exposed to the antibiotic during the binding reaction.

and assaying the binding of non cognate, purified [$^3$H]Tyr-tRNA$^{Tyr}$ to poly(U)-programmed 30S subunits, we verified that this was also the case with neomycin B, kanamycin B and gentamicin C1a. (The incorporation of tyrosine by poly(U)-directed polypeptide synthesizing systems is strongly stimulated by these drugs (13)). On the other hand, 70S ribosomes in the presence of poly(U), EF-T, GTP and antibiotic did bind [$^3$H]Tyr-tRNA. Consequently, we treated either 30S or 50S subunits with 2 μM neomycin B, removed the excess of unbound antibiotic by Sepharose filtration and assayed the binding of [$^3$H]Tyr-tRNA to the treated subunit after supplementation with the complementary untreated subunit. Table II shows that the reassociated 70S ribosomes containing treated 30S subunits bound 66% as much [$^3$H]Tyr-tRNA as did 70S ribosomes assayed in the presence of 2 μM neomycin B. In contrast, those containing treated 50S subunits did not bind [$^3$H]Tyr-tRNA, unless fresh neomycin B was added to the assay mixture. Moreover, the ribosomes containing treated 30S subunits were as active in [$^3$H]Tyr-tRNA binding as 70S ribosomes treated with 2 μM neomycin B and similarly freed of excess antibiotic (Table II), suggesting that the 50S subunit does not influence the binding of antibiotic to the 30S subunit. Similar qualitative results were obtained with 0.1 mM kanamycin B, but the ribosomes containing treated 30S subunits bound only 25% as much [$^3$H]Tyr-tRNA as ribosomes in the presence of 0.1 mM antibiotic (not shown). In another experiment, 0.25 mM gentamicin C1a did not promote binding of [$^3$H]Tyr-tRNA

Table III. Effect of treating ribosomal subunits with neomycin B on the translocation of Ac[$^{14}$C]Phe-tRNA

| Subunit treated before Sepharose filtration | Neomycin B addition during assay | Ac[$^{14}$C]Phe-puromycin formation (pmol) | |
|---|---|---|---|
| | | A | B |
| None | - | 1.62 (100) | 1.20 (100) |
| 30S | - | 1.16 ( 72) | 0.59 ( 49) |
| 30S | + | 0.46 ( 28) | 0.22 ( 18) |
| 50S | - | 1.33 ( 82) | 0.49 ( 41) |
| 50S | + | 0.30 ( 19) | 0.21 ( 18) |

The ribosomal subunits indicated in the Table were treated with either 2 μM (A) or 7 μM (B) neomycin B and the excess antibiotic was removed by Sepharose filtration (Methods). The treated subunits were supplemented with an equimolar amount of untreated complementary subunits and Ac[$^{14}$C]Phe-tRNA was bound in the presence of poly(U), deacylated tRNA$^{Phe}$, and 20 mM magnesium acetate to the A-site of the resulting 70S ribosomes (14). 70S couples containing one treated subunit and control couples containing no treated subunits bound the same amount of Ac[$^{14}$C]Phe-tRNA (0.51-0.66 molecules/ribosome). EF-G plus GTP-dependent Ac[$^{14}$C]Phe-puromycin formation was assayed in mixtures (53-64 μl) containing: 30 mM $NH_4Cl$, 30 mM KCl, 15 mM Tris-HCl pH 7.8, 12 mM magnesium acetate, 3 mM 2-mercaptoethanol, 2 mM dithiothreitol, 5-6 pmol 70S couples with A-site-bound Ac[$^{14}$C]Phe-tRNA, 20 μg/ml EF-G, 0.2 mM GTP, 0.5 mM puromycin and when indicated either 2 μM (A) or 7 μM (B) neomycin B. After incubation at 30° for 5 min Ac[$^{14}$C]Phe-puromycin synthesized was determined (14). Values obtained in controls without EF-G and GTP have been subtracted. Values in brackets indicate percent of remaining translocation activity.

unless the antibiotic was added to the binding assay; gel filtration probably removed all gentamicin C1a bound to misreading-inducing site(s).

*Inhibition of peptidyl-tRNA translocation.* To localize the target site of neomycin B responsible for the inhibition of translocation, reassociated ribosomes containing either 30S or 50S subunits treated with this antibiotic were made to bind Ac[$^{14}$C]Phe-tRNA into their A-site; translocation was then assayed by measuring the EF-G plus GTP-dependent synthesis of Ac[$^{14}$C]Phe-puromycin. Table III shows that treatment of subunits with 2 μM neomycin B caused only weak (20-30%) inhibition of translocation while treatment with 7 μM antibiotic promoted much stronger inhibition (50-60%). In each case the effect was essentially the same regardless of which subunit was treated with the antibiotic.

Discussion. The finding that neomycin B and related antibiotics stabilize EF-G and GDP on isolated 50S subunits (Table I), indicates that these antibiotics interact with this subunit and modify its functional properties. On the other hand, the 30S subunit is probably the target for misreading since its treatment with neomycin B or kanamycin B induces this effect (Table II). It seems unlikely that, during the misreading assay which requires 50S subunits, the antibiotic

is transferred to the 50S subunit to exert its effect there; for under the conditions of our experiment isolated 50S subunits do not bind antibiotic to any misreading-promoting site(s) (Table II) and, consequently, the affinity of the 50S particle for the antibiotic would be expected to increase sharply upon 70S couple formation, in contradiction with the reported similar affinity constants of 50S and 70S particles for [$^3$H]kanamycin (6). However, it should be stressed that the gradual stimulation of misreading with increasing concentrations of gentamicin and kanamycin up to very high concentrations of antibiotic (0.1-1 mM) (2,4) suggests that there may be additional low-affinity sites perhaps on both ribosomal subunits. Moreover, both ribosomal subunits are clearly involved in the expression of misreading since isolated 30S particles, even though they bind antibiotic, fail to misread ((12) and our unpublished observations), and a mutation in 50S protein L6 decreases the capacity for misreading (5).

The inhibition of translocation of AcPhe-tRNA was the same regardless of which ribosomal subunit was treated with neomycin B (Table III). While the simplest explanation is that the interaction of neomycin B with each subunit impairs translocation, the possibility of the antibiotic being exchanged between subunits should be kept in mind. It is of interest that other inhibitors of translocation like the peptide antibiotics viomycin and tuberactinomycin O (15-17) also bind to both ribosomal subunits, and neomycin, kanamycin and gentamicin strongly inhibit the binding of tuberactinomycin O to each subunit (17). Translocation probably involves coordinated rearrangements of both ribosomal subunits and it would not be surprising that distortion of either subunit by an antibiotic might impair the process.

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