Fidelity of the Eukaryotic Codon–Anticodon Interaction: Interference by Aminoglycoside Antibiotics†

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ABSTRACT: A homologous in vitro method was developed from *Tetrahymena* for ribosomal A-site binding of aminoacyl-tRNA to poly(uridylic acid)-programmed ribosomes with very low error frequency. The reaction mixture pH was the crucial factor in the stable A-site association of aminoacyl-tRNA with high fidelity. At a pH greater than 7.1, endogenous activity translocated A-site-bound aminoacyl-tRNA to the P site. If translocation was allowed to occur, a near-cognate aminoacyl-tRNA, Leu-tRNA, could stably bind to the ribosome by translocation to the ribosomal P site. Near-cognate aminoacyl-tRNA did not stably bind to either site when translocation was blocked. Misreading antibiotics stimulated the stable association of near-cognate aminoacyl-tRNA to the ribosomal A site, thereby increasing the error frequency by several orders of magnitude. Ribosome binding of total aminoacyl-tRNA near equilibrium was not inhibited by misreading antibiotics; however, initial rate kinetics of the binding reaction were dramatically altered such that a 6-fold rate increase was observed with paromomycin or hygromycin B. The rate increase was evident with both cognate and near-cognate aminoacyl-tRNAs. Several antibiotics were tested for misreading potency by the ribosome binding method. We found gentamicin G418 > paromomycin > neomycin > hygromycin B > streptomycin in the potentiation of misreading. Tetracycline group antibiotics effectively inhibited A-site aminoacyl-tRNA binding without promoting misreading.

Aminoglycoside antibiotics promote the misinsertion of amino acids into growing polypeptide chains in eukaryotes, as well as in prokaryotes [see Vazquez (1979)]. Many aminoglycosides have a high specificity in their effect on different organisms (Vazquez, 1979); a classic demonstration was the failure of streptomycin (SM)[1] to affect cytoplasmic protein synthesis in a variety of eukaryotes [see review by Vasquez et al., (1982)]. Ribosomes play a crucial role in the fidelity of protein synthesis; error-promoting antibiotics and ribosomal mutants have been pivotal in the study of this process. Direct experimental evidence in support of a GTP-dependent kinetic proofreading model in *Escherichia coli* was recently established (Thompson et al., 1981a; Thompson & Dix, 1982); the aminoglycoside streptomycin was shown to inhibit this process (Thompson et al., 1981b). The influence of aminoglycoside antibiotics on fidelity in eukaryotes has not been characterized in such detail, but the phenomenon of misreading by some of these drugs was demonstrated in crude systems derived from yeast (Vazquez et al., 1982; Palmer et al., 1979a), wheat germ (Wilhelm et al., 1978a), mammalian cells (Wilhelm et al., 1978b), and *Tetrahymena* (Palmer & Wilhelm, 1978). Furthermore, in vivo drug-induced nonsense suppression was observed in yeast (Singh et al., 1979; Palmer et al., 1979b). The most potent phenotypic suppressor was paromomycin, but the structurally dissimilar aminoglycoside hygromycin B also had activity. Some aminoglycoside antibiotics also demonstrated strong inhibitory effects on the elongation cycle of protein synthesis (Vasquez, 1978). Inhibition of protein synthesis by these drugs interferes with interpretation of the misreading effect, as pointed out by Vasquez et al. (1982). To study drug-induced infidelity and the pleotrophic effects of aminoglycosides on elongation independently, we developed a homologous in vitro aminoacyl-tRNA binding assay from *Tetrahymena thermophila*. This eukaryote was chosen for study because it is highly sensitive to growth inhibition by certain aminoglycosides (Palmer & Wilhelm, 1978). Furthermore, *T. thermophila* mutants resistant to hygromycin B and/or paromycin have been isolated and are ribosomal mutants (J. M. Wilhelm, unpublished results).

The binding method described here is superior to the polymerization methods used until now because it allowed the use of very low Mg^{2+} concentrations; consequently, Mg^{2+}-induced infidelity could be reduced dramatically [see Laughrea (1981)]. Hygromycin B (HM B) and paromomycin (PM) stimulated the initial rates of both near-cognate (Leu) and cognate (Phe) aminoacyl-tRNA binding to poly(U)-programmed ribosomes, whereas SM had no effect. Paromomycin-promoted near-cognate aminoacyl-tRNA binding to ribosomes in the presence of elongation factor 1 (EF1) or high Mg^{2+} concentration demonstrated that ribosomes, not elongation factors, were the specific target of drug action. Among the antibiotics tested, we found gentamicin G418 (G418) and PM were the strongest inducers of misreading, HM B and neomycin (NM) were modest inducers, and SM was totally inactive. Tetracycline and minocycline did not promote misreading; however, tetracycline and minocycline effectively inhibited aminoacyl-tRNA binding to *Tetrahymena* ribosomes and inhibited growth.

Experimental Procedures

Materials. Hygromycin, paromomycin, and gentamicin G418 (potency of the monosulfate; 460 μg/mg) were generous gifts of Eli Lilly, Parke Davis and Co., and Schering Corp., respectively; all other antibiotics were from Sigma.

Strain. Tetrahymena thermophila strain B1868 was grown as described (Palmer & Wilhelm, 1978) except that the growth medium also contained 0.003% chelated iron.

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[1] Abbreviations: PM, paromomycin; HM B, hygromycin B; G418, gentamicin G418; NM, neomycin; SM, streptomycin; EF1, eukaryotic elongation factor 1; EF2, eukaryotic elongation factor 2; poly(U), poly(uridylic acid); Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, disodium ethylenediaminetetraacetate; DTT, dithiothreitol; Cl_3CCOOH, trichloroacetic acid; tRNA, transfer ribonucleic acid; Phe-tRNA, phenylalanyl-$tRNA^{Phe}$; Leu-tRNA, leucyl-$tRNA^{Leu}$; 2-DOS, 2-deoxystreptamine; HA, hydroxylapatite; SDS, sodium dodecyl sulfate.

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Preparation of tRNA. Batch cultures of 5 L were grown with forced aeration to a cell density of 200 000 cells/mL. Cells were extracted with phenol by the method of Kuchino et al. (1981) except that 1 mM 2-mercaptoethanol was included in the buffer. Ethanol-precipitated tRNA was further purified by DEAE-cellulose chromatography by the method of Keegan & Berech (1979) and then deacylated as described by Eustice et al. (1981).

Preparation of Cytoplasmic Extracts and the Supernatant Fraction. Cell extracts were prepared by the method of Palmer & Wilhelm (1978) except that (1) S-30 supernatants were centrifuged at 100000*g* for 90 min, and the top two-thirds were taken as S-100, (2) supernatants prepared for use in aminoacylation reactions were not preincubated, and (3) preincubation was performed in the presence of 1.0 mM puromycin when the S-30 was used as a source for ribosomes.

Purification of 80S Ribosomes. Ribosomes were pelleted from S-30 supernatants by centrifugation at 100000*g* for 90 min and resuspended in G-25 column buffer (Palmer & Wilhelm, 1978). Approximately 200–300 A_{260} units were loaded onto 34-mL sucrose gradients (10–30% sucrose, prepared in 20 mM Tris-HCl, pH 7.6, 4.0 mM $MgCl_2$, 100 mM KCl, and 1.0 mM 2-mercaptoethanol). The monosome peaks were collected from the gradients after centrifugation at 37000*g* for 18 h. Monosomes were pelleted at 100000*g* for 5–6 h, resuspended in 18 mM Hepes–KOH, pH 7.6, 3.6 mM magnesium acetate, 90 mM KCl, 5.0 mM 2-mercaptoethanol, and 10% glycerol, and stored at −70 °C.

Purification of Elongation Factors. Starting with the supernatant fraction, we separated EF1 and EF2 by hydroxylapatite chromatography as described by Kemper & Merrick (1976). Fractions containing EF1 were located by stimulation of [^{3}H]Phe-tRNA binding to poly(U)-programmed ribosomes; fractions containing EF2 were located by stimulation of polyphenylalanine synthesis in the presence of EF1. One unit of EF1 is the amount required to bind 1 pmol of Phe-tRNA to ribosomes. Alternatively, EF1 was resolved from EF2 by passage through a column of DEAE-Bio-Gel equilibrated with 20 mM Tris-HCl, pH 7.6, 50 mM KCl, 1.0 mM DTT, 0.1 mM phenylmethanesulfonyl fluoride, and 25% glycerol. These preparations were used as a source of EF1 in the experiments of Figure 2 and Tables VI and VII.

Polyphenylalanine Synthesis. Polyphenylalanine synthesis was assayed in 100-μL reaction mixtures which contained 20 mM Hepes–KOH, pH 7.6, 10 mM magnesium acetate, 1.0 mM DDT, 0.25 mM GTP, 100 mM KCl, 4.0 pmol of 80S ribosomes, 12.8 pmol of [^{14}C]Phe-tRNA, 100 μg/mL poly(U), 10 μL of EF1, and 10 μL of EF2. After 30 min at 30 °C, 90 μL was removed for the determination of hot Cl_3CCOOH-precipitable incorporation as previously described (Palmer & Wilhelm, 1978).

Aminoacylation of tRNA. Aminoacylation of tRNA was performed at 30 °C in reaction mixtures containing 50 mM Hepes–KOH, pH 7.6, 10 mM magnesium acetate, 70 mM KCl, 1.25 mM ATP, 30 μM CTP, 10 mM creatine phosphate, 50 μg/mL creatine phosphokinase, 3.0 mM DTT, 5.0 μM each of amino acids (one or two labeled), 24–60 μg/mL of S-100 protein, and 12 A_{260} units/mL tRNA. Products were processed as described by Eustice et al. (1981). Counting efficiencies were compensated for by the method of Albright et al. (1978).

Ribosome Binding Assays. Reaction mixtures contained 20 mM Hepes–KOH, 3.1 mM magnesium acetate, 21 mM KCl (50 mM K^+), 0.7 mM GTP, 3.0 pmol of [^{14}C]Phe-tRNA, 3.0 pmol of ribosomes, 10 units of EF1, and 100 μg/mL poly(U), final pH 6.9 (or as indicated in the figure legends). Reactions were incubated at 30 °C for 10 min and then diluted with 2.0 mL of ice-cold 20 mM Hepes–KOH, 3.1 mM magnesium acetate, and 50 mM KCl (the pH always matched that of the reaction mixture). Samples were filtered through Millipore-type HA filters and washed twice with 2.0 mL of cold dilution buffer. Filters were dried and then counted in 5.0 mL of Econofluor (NEN).

Table I: Dependence of Polyphenylalanine Synthesis on EF1 and EF2

additions	[^{14}C]Phe incorporated	
	pmol	% of complete
EF1	0.18	4.2
EF2	0.31	7.2
EF1 + EF2 (complete)	4.30	100.0[a]

[a] Represents 33.6% of the [^{14}C]Phe-tRNA in the reaction mixture.

Puromycin Reaction. Estimation of puromycin-reactive aminoacyl-tRNA bound to ribosomes was determined as described by Eustice et al. (1981). To determine A-site-bound aminoacyl-tRNA, duplicate reaction mixtures were concomitantly assayed for the total bound radioactivity and the puromycin-reactive radioactivity. The difference was taken as the A-site-bound aminoacyl-tRNA.

Results

Aminoacylation of tRNA. The addition of 30 μM CTP gave a 50–70% stimulation of phenylalanine-specific acceptance, suggesting (1) that a significant proportion of the tRNA was cleaved at the 3′ end and (2) that *Tetrahymena* S100 contained a terminal nucleotide transferase capable of regenerating CCA termini. Typical product analysis demonstrated relative acceptor activities of 1:4:7.4:8 for Met:Phe:Leu:Lys. In all ribosome binding experiments, unfractionated aminoacyl-tRNA was used.

Purification and Properties of Elongation Factors. Hydroxylapatite-purified EF1 and EF2 were tested for cross-contamination by assaying the dependence of polyphenylalanine synthesis on each factor. As shown in Table I, polyphenylalanine synthesis was highly dependent on the presence of both factors. Treatment of EF1 with either GDP or $GDP(CH_2)P$ greatly inhibited activity in the binding of Phe-tRNA to ribosomes. This inhibition could not be overcome by the addition of excess GTP, suggesting (1) that a GDP–GTP exchange factor was not present and (2) that these purification conditions selected for preformed EF1–GTP complexes. SDS gel electrophoresis of HA-purified EF1 revealed three main bands of Coomassie-stainable material that ranged in molecular weight from 47 000 to 58 000. Electrophoresis of hydroxylapatite-purified EF2 revealed a major band at 97 000 daltons. DEAE-Bio-Gel-purified EF1 contained more impurities than hydroxylapatite-purified EF1 as judged by SDS gel electrophoresis but contained one major band at 48 000 daltons.

Precise Conditions for A-Site-Specific Binding. To obtain optimal conditions for exclusive A-site binding of cognate tRNA and maximal discrimination against noncognate tRNA, we tested the effects of salts and pH on the binding of Phe-tRNA and Leu-tRNA to poly(U)-programmed ribosomes. First, concentrations of potassium ion above 60 mM were very inhibitory (Figure 1A). All subsequent experiments were performed at 50 mM K^+. Second, high magnesium ion concentration promotes the binding of aminoacyl-tRNA to *E. coli* ribosomes in the absence of EF-Tu (Peters & Uarus, 1979)

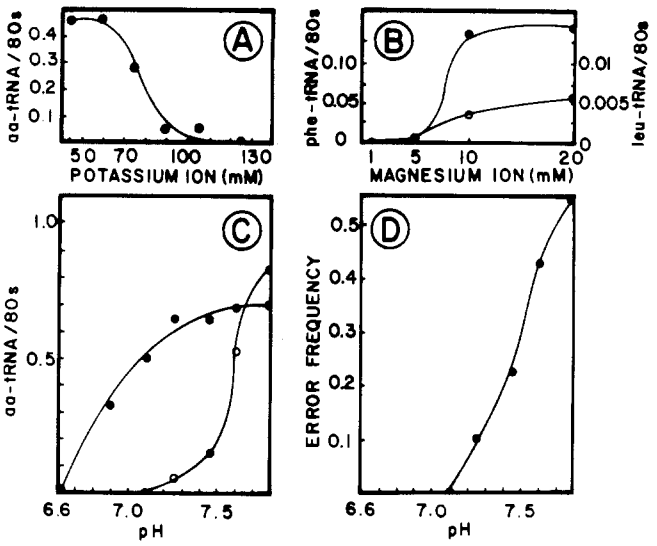

FIGURE 1: Effect of K^+, Mg^{2+}, and pH on the binding reaction. (A) Effect of K^+ on the binding of Phe-tRNA at pH 7.18. Reaction mixtures (25 μL) contained 1.0 pmol of ribosomes, 1.25 pmol of [^{14}C]Phe-tRNA, 1.22 units of HA-purified EF1, and 3.1 mM Mg^{2+}. KCl was added to the reaction mixture, and binding was determined after 10 min at 30 °C. (B) Effect of Mg^{2+} on the binding of Phe-tRNA and Leu-tRNA in the absence of EF1. Reaction mixtures (50 μL) contained 3.0 pmol of [^{14}C]Phe-tRNA, 8.75 pmol of [^{3}H]Leu-tRNA, 3.0 pmol of ribosomes, and 50 mM K^+, with a final pH of 6.9. Binding was determined after 10 min at 30 °C. (C) Effect of pH on the binding of Phe-tRNA and Leu-tRNA. Reaction mixtures (50 μL) contained 2.0 pmol of [^{14}C]Phe-tRNA, 5.83 pmol of [^{3}H]-Leu-tRNA, 2.0 pmol of ribosomes, 22.5 units of HA-purified EF1, 3.1 mM Mg^{2+}, and 50 mM K^+. The final pH was determined with a pH electrode in oversized reaction mixtures which contained all buffer salts and other components except ribosomes, aminoacyl-tRNA, and EF1. The pH was varied by changing the pH of the Hepes buffer, and then the K^+ concentration was brought to 50 mM with KCl. Binding was determined after 10 min at 30 °C. (D) Plot of the error frequency as a function of pH. Data from (C) were transformed as described under Experimental Procedures. All data were corrected for controls lacking poly(U). (●) [^{14}C]Phe-tRNA; (○) [^{3}H]Leu-tRNA.

or in the absence of EF1 with wheat ribosomes (Golinska & Legocki, 1973). To minimize interference from nonenzymatic binding, we determined the concentration of magnesium which gives total dependence on EF1. Figure 1B demonstrates that concentrations of magnesium ion above 5 mM stimulate Phe-tRNA and Leu-tRNA binding to ribosomes in the absence of EF1. Lastly, the final pH of the reaction mixture had a dramatic effect on the discrimination between Phe-tRNA and Leu-tRNA (Figure 1C). Initially, it appeared that the binding fidelity was inversely proportional to pH (Figure 1D). A pH greater than 7.1 promoted significant binding of Leu-tRNA to poly(U)-programmed ribosomes although the binding of lysyl-tRNA (a distantly related codon) was not detectable (detection level of 0.1 pmol). Subsequent analysis of aminoacyl-tRNA distribution between A and P sites revealed striking differences when the pH was changed (Table II). At pH 7.5, Phe-tRNA was distributed between both sites, whereas Leu-tRNA was exclusively associated with the P site. There were a few exceptions to the exclusive P-site association of labeled leucine, however. Atypical binding conditions which favored a second round of aminoacyl-tRNA binding (e.g., excessive aminoacyl-tRNA concentrations) resulted in measurable leucine associated with the A site. This A-site-bound leucine probably represents a Leu-Phe dipeptide attached to A-site-bound $tRNA^{Phe}$. At pH 6.9, Phe-tRNA was only found in the A site, and Leu-tRNA was barely detectable in either site. The stable association of aminoacyl-tRNA with the P site at

Table II: Distribution of Aminoacyl-tRNA between A and P Sites at pH 6.9 and 7.5[a]

aminoacyl-tRNA	site[b]	pmol bound at pH 6.90	pmol bound at pH 7.5
[^{14}C]Phe-tRNA	A + P	0.64	1.30
	A	0.55	0.82
	P	0.09	0.48
[^{3}H]Leu-tRNA	A + P	0	0.37
	A	0	0
	P	0.02[c]	0.51[c]

[a] Final pH of the reaction mixture. Reaction mixtures were as described in the legend to Figure 1C. [b] Determined as described under Experimental Procedures. [c] Frequently, P-site values slightly exceeded the level of binding found by the filtration method because of the longer incubation time used during puromycin treatment.

Table III: Effect of Inhibitors of Translocation on Aminoacyl-tRNA Binding to the P Site at pH 7.6[a]

additions	pmol reactive with puromycin [^{14}C]Phe-tRNA	[^{3}H]Leu-tRNA
none	0.318	0.047[b]
100 μM cycloheximide	0	0.020
10 μM emetine	0	0.007
100 μM emetine	0	0

[a] Reaction mixtures contained 3.0 pmol of ribosomes, 2.0 pmol of [^{14}C]Phe-tRNA (4.52 pmol of [^{3}H]Leu-tRNA), and 7.5 units of HA-purified EF1. [b] This value was lower than that in the experiment described in Table II because the ribosomes were in excess in this experiment.

high pH was blocked by inhibitors of translocation (Table III). Cycloheximide inhibition of translocation could be partially overcome by the addition of EF2. Inhibition by 10 μM emetine could be fully overcome by the addition of EF2 (data not shown). The activity of these inhibitors on EF2-dependent translocation is consistent with their mechanism of action in other in vitro systems (Barbacid et al., 1975; Vazquez, 1979). These results suggest that (1) although EF1 alone does not support polyphenylalanine synthesis, sufficient endogenous activity was present to translocate aminoacyl-tRNA (at a pH greater than 7.1) and (2) Leu-tRNA could enter the A site and stably bind to ribosomes by translocation to the P site. Translocation of near-cognate aminoacyl-tRNA under these conditions may, in part, be an artifact of a vacant P site. This should not occur during polypeptide synthesis since the P site would be occupied by peptidyl-tRNA. Support for this hypothesis was that polyphenylalanine synthesis at high and low pH showed less than 2-fold variation in fidelity (S. Skelly and J. M. Wilhelm, unpublished results). To study the fidelity of the selection process in vitro, the manipulation of pH becomes a useful tool to selectively bind amionoacyl-tRNA to the A site, a method with a distinct advantage over the use of chemical translocation inhibitors.

The results in Table IV demonstrate the dependence of the binding reaction on the addition of template, ribosomes, and EF1. The values shown for the complete reaction mixture were not corrected for any control values. The Leu-tRNA binding observed was equal to the Leu-tRNA binding found in each type of control reaction mixture. Therefore, the deletion of any factor in the reaction mixture would yield an appropriate control value, resulting in Leu-tRNA binding below the level of detection. Error frequencies of 1/150 were not detectable with the specific activity of [^{3}H]leucine used in these experiments. (Detection levels were determined by 95% confidence

Table IV: Factor Dependence of the Binding Reaction at pH 6.9[a]

deletion	aminoacyl-tRNA	pmol bound[b] A + P site	A site	P site
none (complete)	[^{14}C]Phe	1.22 ± 0.07	1.14 ± 0.09	0.08 ± 0.02
	[^{3}H]Leu	0.043 ± 0.005	0 ± 0.009	0.047 ± 0.004
poly(U)	[^{14}C]Phe	0.04 ± 0.01	0.01 ± 0.01	0.03 ± 0.001
	[^{3}H]Leu	0.037 ± 0.003	0 ± 0.004	0.043 ± 0.001
ribosomes	[^{14}C]Phe	0.03 ± 0.01	0 ± 0.02	0.03 ± 0.01
	[^{3}H]Leu	0.024 ± 0.016	0 ± 0.017	0.041 ± 0.001
EF1	[^{14}C]Phe	0.08 ± 0.01	0.05 ± 0.02	0.03 ± 0.01
	[^{3}H]Leu	0.053 ± 0.019	0.011 ± 0.021	0.042 ± 0.002

[a] Reaction mixtures contained 3.0 pmol of ribosomes, 3.0 pmol of [^{14}C]Phe-tRNA (8.75 pmol of [^{3}H]Leu-tRNA), and 10 units of HA-purified EF1. [b] Values shown are the mean of triplicate determinations ± 1 standard deviation.

Table V: Effect of Antibiotics on the Growth of *Tetrahymena* Strain B1868

antibiotic	concn (μM) required for complete growth inhibition[a]
PM	70
G418	30
HM B	85
SM	>5000[b]
NM	>>5000[c]
Tc[d]	312
Mc[e]	101

[a] Single cells were transferred to drops containing various drug concentrations. Clone sizes were estimated 24 h later; in drug-free controls, the size was consistent with seven to eight population doublings. The concentrations reported prevented all increase in cell numbers. [b] This concentration reduced growth to 4.5 population doublings in 24 h. [c] This was the highest concentration tested, and it gave no growth inhibition. [d] Tc is tetracycline. [e] Mc is minocycline.

Table VI: Effect of Tetracycline Group Antibiotics on the Binding Reaction[a]

antibiotic	concn (μM)	pmol of [^{14}C]Phe-tRNA bound
none	0	0.58
tetracycline	10	0.58
	100	0.30
	500	0
minocycline	10	0.56
	100	0.33
	500	0

[a] Reaction mixtures contained 3.0 pmol of [^{14}C]Phe-tRNA, 3.0 pmol of ribosomes, and 2.7 units of DEAE-Bio-Gel-purified EF1.

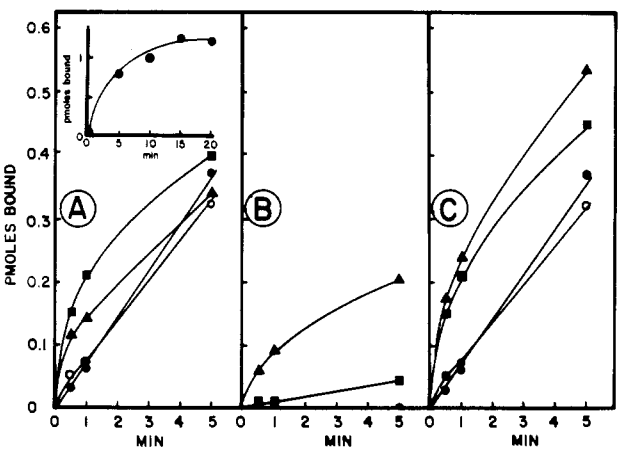

FIGURE 2: Effect of aminoglycosides on the initial rates of the binding reaction. All reaction mixtures (50 μL) contained 3.0 pmol of [^{14}C]Phe-tRNA, 2.19 pmol of [^{3}H]Leu-tRNA, 3.0 pmol of ribosomes, 2.7 units of DEAE-Bio-Gel-purified EF1, 3.1 mM Mg^{2+}, 50 mM K^{+}, and 10 μM samples of the aminoglycosides indicated. (A) Binding of Phe-tRNA; (B) binding of Leu-tRNA; (C) binding of Phe-tRNA plus Leu-tRNA; (●) no additions; (▲) 10 μM PM; (■) 10 μM HM B; (○) 10 μM SM. Inset: Typical time course at 30 °C. Reaction mixtures were the same as described for panels A–C except 8.2 units of DEAE-Bio-Gel-purified EF1 were present.

intervals from a Student's *t* test distribution.) These conditions could be used with larger reaction volumes and/or higher specific activities of near-cognate amino acids to obtain reliable basal error frequencies. For the purpose of studying drug-induced misreading of codons, limits of detection <1/150 substitutions per binding event were more than adequate.

Effects of Tetracycline Antibiotics on Aminoacyl-tRNA Binding. Tetracycline and minocycline were found to effectively inhibit the growth of *Tetrahymena* (Table V). Table VI demonstrates the activity of tetracycline and minocycline on the A-site binding of Phe-tRNA. Both antibiotics had similar dose responses, where binding was inhibited about 50% by a 100 μM sample of either drug. No misreading was observed with either tetracycline or minocycline.

Effects of Aminoglycoside Antibiotics on Aminoacyl-tRNA Binding. In the absence of drugs, the reaction was nearly complete by 10 min (Figure 2, inset). Preliminary experiments with limiting ribosome concentrations and Phe-tRNA suggested that the net binding of Phe-tRNA near the equilibrium value was inhibited by PM, although initial rates were stimulated. SM, an aminoglycoside antibiotic which does not stimulate misreading with eukaryotic ribosomes, had no effect on the time course of binding with *Tetrahymena* ribosomes (Figure 2). Further examination of the reaction with [^{14}C]-Phe-tRNA and [^{3}H]Leu-tRNA demonstrated that Leu-tRNA had simply replaced Phe-tRNA in ribosome binding (Figure 2A,B). Supporting evidence for a simple replacement was that inhibition of Phe-tRNA binding did not occur when ribosomes were in excess over aminoacyyl-tRNA concentrations. The summation of Phe-tRNA and Leu-tRNA bound to ribosomes over the time course is shown in Figure 2C. The net amount of aminoacyl-tRNA (Leu and Phe) bound at early time points showed that initial rates of net aminoacyl-tRNA binding were stimulated nearly 6-fold. Similar results were also obtained with HM B. The coincidence of misreading and initial rate stimulation suggests that these parameters might be coupled. The binding of [^{3}H]Leu-tRNA to poly(U)-programmed ribosomes as a function of PM concentration is shown in Figure 3. All bound Leu-tRNA was at the A site as estimated by the puromycin reaction (PM did not inhibit this reaction). The binding of Leu-tRNA in the presence of EF1 in low Mg^{2+} concentrations (Figure 3A) required a PM concentration equal to or in excess of the ribosome concentration. Binding of Leu-tRNA was maximal at 10 μM (data not shown), and the error frequency [picomoles of Leu/(picomoles of Leu + picomoles of Phe)] was 0.36 at 10 μM PM (Figure 3B). The

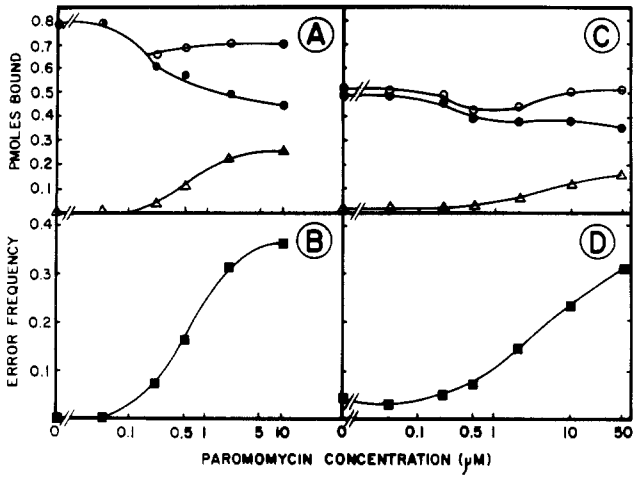

FIGURE 3: Dose response of aminoacyl-tRNA binding with PM. (A) EF1-mediated binding of aminoacyl-tRNA as a function of PM concentration. Reaction mixtures contained 3.0 pmol of [^{14}C]Phe-tRNA, 6.77 pmol of [^{3}H]Leu-tRNA, 3.0 pmol of ribosomes, 10 units of HA-purified EF1, 3.1 mM Mg^{2+}, and 50 mM K^{+} with a final pH of 6.90. Binding was determined after 10 min at 30 °C. (B) Data from (A) transformed into error frequencies. (C) Magnesium-mediated binding of aminoacyl-tRNA as a function of PM concentration. Reaction mixtures contained 3.0 pmol of [^{14}C]Phe-tRNA, 5.57 pmol of [^{3}H]Leu-tRNA, 3.0 pmol of ribosomes, 20 mM Mg^{2+}, and 50 mM K^{+}, and the final pH was 6.90. Binding was determined after 10 min at 30 °C. (D) Data from (C) transformed into error frequencies. (●) [^{14}C]Phe-tRNA; (▲) [^{3}H]Leu-tRNA; (○) [^{14}C]Phe-tRNA plus [^{3}H]Leu-tRNA.

error frequency in some experiments with 10 μM PM approached 0.5, indicating that ribosome capacity to discriminate between cognate and near-cognate aminoacyl-tRNA was abolished by PM. Wilhelm et al. (1978a) reported on the pattern of misincorporation promoted by PM in the translation of poly(U) by wheat ribosomes. They showed that at 28 μM PM, misincorporation of leucine was by far the most frequent error (equal to about 80% of the phenylalanine incorporation) followed by isoleucine (4-fold less frequent than leucine) and serine (25-fold less frequent than leucine). Lysine incorporation was not detectably stimulated by PM. Therefore, lysyl-tRNA should not respond to PM in the binding reaction. As predicted, lysyl-tRNA did not respond to 10 μM PM (data not shown). Under our usual conditions, there was little or no aminoacyl-tRNA binding in the absence of EF1 (Table IV). However, high concentrations of magnesium ion will promote factor-independent binding of aminoacyl-tRNA (see Figure 1). At 20 mM magnesium acetate, Phe-tRNA was bound, although less efficiently than with EF1 at lower magnesium acetate concentrations (Figure 3C); in addition, significant binding of Leu-tRNA was observed. At 20 mM magnesium acetate, PM-stimulated misreading paralleled the pattern obtained with EF1 (Figure 3C). The concentration of PM required to obtain maximal misreading was higher in the presence of 20 mM magnesium acetate, suggesting that magnesium ions may partially shield ribosomes from the cationic PM (Figure 3C). This effect of high magnesium ion concentration on binding fidelity is consistent with the compiled results obtained from many polypeptide synthesis studies [reviewed by Laughrea (1981)]. A survey of the aminoglycoside antibiotics (Table VII) shows their effectiveness in error production. Gentamicin G418 and PM were the most potent misreaders, HM B and NM were intermediate misreaders, and SM was inactive. Gentamicin G418, PM, and HM B were potent inhibitors of *Tetrahymena* growth; in contrast, NM and SM were very ineffective (Table V). The in vitro potency of these antibiotics did not always correlate

Table VII: Effect of Various Aminoglycosides on the Fidelity of the Binding Reaction at pH 6.9[a]

additions	concn (μM)	error frequency[b]
none	0	0
PM	1.0	0.203
	10.0	0.403
HM B	1.0	0.060
	10.0	0.091
NM	1.0	0.078
	10.0	0.176
G418	1.0	0.385
	10.0	0.408
SM	1.0	0
	10.0	0

[a] Reaction mixtures contained 3.0 pmol of ribosomes, 3.0 pmol of [^{14}C]Phe-tRNA (8.75 pmol of [^{3}H]Leu-tRNA), and 2.7 units of DEAE-Bio-Gel-purified EF1. [b] The error frequency was determined as picomoles of [^{3}H]Leu-tRNA/(picomoles of [^{3}H]Leu-tRNA + picomoles of [^{14}C]Phe-tRNA).

with the in vivo misreading data. A likely explanation is that many aminoglycosides have multiple effects in vivo such as inhibition of initiation and inhibition of chain elongation [see reviews by Davis et al. (1974), Laughrea (1981), and Vazquez et al. (1982)].

Discussion

The assay method described here was sensitive and powerful in the study of aminoacyl-tRNA binding to the A site of the ribosome uncoupled from other reactions of protein synthesis. By careful manipulation of the ionic environment and the pH, error frequencies less than 1/150 have been obtained for the selection of cognate aminoacyl-tRNA (Phe-tRNA) over noncognate aminoacyl-tRNA (Leu-tRNA). The binding of a noncognate aminoacyl-tRNA (lysyl-tRNA) could not be demonstrated in this system, even in the presence of misreading antibiotics. The fidelity of the binding reactions has been extensively studied in prokaryotes [see Thompson & Karim (1982) and references cited therein]. However, there have been few studies on the fidelity of the binding reaction with eukaryotic ribosomes. Igarashi et al. (1982) investigated the fidelity of the binding reaction with wheat ribosomes. Using three partially purified isoaccepting species of Leu-tRNA and purified EF1, they found high levels of binding of each isoacceptor to poly(U)-programmed ribosomes; they did not report if the binding was A site or P site.

Using the method reported here, we found that A-site aminoacyl-tRNA binding was reduced 50% by 100 μM either of tetracycline or of minocycline, consistent with the mechanism of action in prokaryotes [see review by Vazquez (1979)]. Tetracyclines inhibit eurakyotic translation (Franklin, 1963) and have been shown to bind to rat liver ribosomes (Reboud et al., 1982). However, not until now has the mechanism of action been demonstrated in a eukaryote. Error-stimulating aminoglycoside antibiotics also appeared to inhibit cognate aminoacyl-tRNA binding to the A site. However, ribosomes reacted with error-producing drugs had a reduced discrimination between cognate and near-cognate aminoacyl-tRNA; hence, the apparent inhibition of Phe-tRNA binding is interpreted as Leu-tRNA competition with Phe-tRNA for the ribosome A site. Summation of Phe- and Leu-tRNAs bound revealed that no significant binding inhibition occurred with PM, HM B, NM, or G418.

Aminoglycoside antibiotics active in promoting the binding of Leu-tRNA to poly(U)-programmed ribosomes also stimulated the initial rates of the binding reaction. The fact that total aminoacyl-tRNA bound near equilibrium did not sig-

nificantly differ from control values suggests that the initial rate stimulation could not be accounted for by drug-induced activation of "inactive ribosomes". Furthermore, it is unlikely that this rate stimulation was due to a nonspecific effect of these drugs, since the structurally related aminoglycoside SM did not elicit a rate stimulation or misreading. The following three cases might explain the effect on initial rates: (1) a stabilization of the bound products from dissociation; (2) an inhibition of putative proofreading, where a reduced number of normally aborted complexes would become stably bound products; and (3) an increase in the rates of other reactions prior to the proofreading step.

Comparison of the effects of several aminoglycoside antibiotics at two concentrations on the fidelity of the binding reaction (Table VII) allows construction of a rank order based on misreading potency. The most potent was G418 > PM > NM > HM B > SM, where SM did not misread.

Paromomycin (PM), G418, and HM B are all 2-deoxystreptamine (2-DOS) containing antibiotics: members of this class promote various degrees of misreading on cytoplasmic (80S) ribosomes from eukaryotes (Palmer et al., 1979; Wilhelm et al., 1978a,b; Palmer & Wilhelm, 1978). 2-Deoxystreptamine itself does not stimulate any misreading in a polymerizing system from wheat or *Tetrahymena* (J. M. Wilhelm, unpublished results). Paromomycin, neomycin, and G418 are disubstituted 2-DOS molecules with substitutions on the 4,5- or 4,6-positions. Of the disubstituted molecules, PM and G418 fall into the subclass where the 4-substituent is a 2′-monoamino sugar: these molecules stimulate more misreading than drugs where the 4-substituent is a 2′,6′-diamino sugar. Hygromycin B, a monosubstituted 2-DOS, had lower misreading stimulation than PM or G418 and even lower than NM (data shown here). Since NM had misreading activity yet growth was not inhibited, misreading activity alone cannot explain the potent growth inhibition by PM. Furthermore, HM B, with its rather low misreading activity, was a potent growth inhibitor (Table V), again suggesting that misreading is not the only toxic event but that the other effects of aminoglycosides on translation are crucial to toxicity.

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Registry No. G418, 83855-92-9; PM, 7542-37-2; NM, 1404-04-2; HM B, 31282-04-9; SM, 57-92-1.

References

Albright, E. B., Nowak, T. S., & Munro, H. N. (1978) *Anal. Biochem. 91*, 258.

Barbacid, M., Fresno, M., & Vazquez, D. (1975) *J. Antibiot. 28*, 453.

Davis, B. D., Tai, P.-C., & Wallace, B. J. (1974) in *Ribosomes* (Nomura, M., Tissieres, A., & Lengyel, P., Eds.) p 771, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Eustice, D. C., Kull, F. J., & Shrift, A. (1981) *Plant Physiol. 67*, 1054.

Eustice, D. C., Skelly, S., & Wilhelm, J. M. (1983) *Annu. Meet. Am. Soc. Microbiol., 83rd*, 158.

Franklin, T. J. (1963) *Biochem. J. 87*, 449.

Golinska, B., & Legocki, A. B. (1973) *Biochim. Biophys. Acta 324*, 156.

Igarashi, K., Hashimoto, S., Miyake, A., Kashiwagi, K., & Hirose, S. (1982) *Eur. J. Biochem. 128*, 594.

Keegan, F. P., & Berech, J. (1979) *J. Protozool. 26*, 502.

Kemper, W. M., & Merrick, W. C. (1976) *Arch. Biochem. Biophys. 174*, 603.

Kuchino, Y., Mita, T., & Nishimura, S. (1981) *Nucleic Acids Res. 9*, 4557.

Laughrea, M. (1981) *Biochimie 63*, 145.

Palmer, E., & Wilhelm, J. M. (1978) *Cell (Cambridge, Mass.) 13*, 329.

Palmer, E., Wilhelm, J. M., & Sherman, F. (1979a) *J. Mol. Biol. 128*, 107.

Palmer, E., Wilhelm, J. M., & Sherman, F. (1979b) *Nature (London) 277*, 148.

Peters, M., & Yarus, M. (1979) *J. Mol. Biol. 134*, 471.

Reboud, A.-M., Dubost, S., & Reboud, J.-P. (1982) *Eur. J. Biochem. 124*, 389.

Singh, A., Ursic, D., & Davies, J. (1979) *Nature (London) 277*, 146.

Thompson, R. C., & Dix, D. B. (1982) *J. Biol. Chem. 257*, 6677.

Thompson, R. C., & Karim, A. M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4922.

Thompson, R. C., Dix, D. B., Gerson, R. B., & Karim, A. M. (1981a) *J. Biol. Chem. 256*, 81.

Thompson, R. C., Dix, D. B., Gerson, R. B., & Karim, A. M. (1981b) *J. Biol. Chem. 256*, 6676.

Vazquez, D. (1978) *Int. Rev. Biochem. 18*, 169.

Vazquez, D. (1979) *Mol. Biol., Biochem. Biophys. 30*, 1.

Vazquez, D., Zaera, E., Dolz, H., & Jimenz, A. (1982) in *Protein Biosynthesis in Eukaryotes* (Perez-Bercoff, R., Ed.) p 311, Plenum Press, New York.

Wilhelm, J. M., Pettitt, S. E., & Jessop, J. J. (1978a) *Biochemistry 17*, 1143.

Wilhelm, J. M., Jessop, J. J., & Pettitt, S. E. (1978b) *Biochemistry 17*, 1149.