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Dissecting the Ribosomal Inhibition Mechanisms of Edeine and Pactamycin: The Universally Conserved Residues G693 and C795 Regulate P-Site RNA Binding

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Summary

The crystal structures of the universal translation-initiation inhibitors edeine and pactamycin bound to ribosomal 30S subunit have revealed that edeine induces base pairing of G693:C795, residues that constitute the pactamycin binding site. Here, we show that base pair formation by addition of edeine inhibits tRNA binding to the P site by preventing codon-anticodon interaction and that addition of pactamycin, which rebreaks the base pair, can relieve this inhibition. In addition, edeine induces translational misreading in the A site, at levels comparable to those induced by the classic misreading antibiotic streptomycin. Binding of pactamycin between residues G693 and C795 strongly inhibits translocation with a surprising tRNA specificity but has no effect on translation initiation, suggesting that reclassification of this antibiotic is necessary. Collectively, these results suggest that the universally conserved G693:C795 residues regulate tRNA binding at the P site of the ribosome and influence translocation efficiency.

Introduction

Edeine and pactamycin are representatives of a small subset of universal translation inhibitors, both proposed to target the initiation phase of protein synthesis (reviewed by Gale et al., 1981). Edeine A1 (termed Ede hereafter) has been shown to specifically inhibit binding of aminoacyl-tRNAs to the P site of both 30S subunits and 70S ribosomes, as well as to eukaryotic 40S subunits. Consistently, the P-site specificity of Ede is exemplified by the differential effects of Ede on the direct binding of particular internal ribosome entry site (IRES) containing viral RNAs, i.e., Ede strongly inhibits translation of IRES containing viral RNAs that initiate out of the P site in an Met-$tRNA_i$-dependent manner but has no effect on that of the cricket paralysis virus (CrPV) RNA that initiates from the A site (Wilson et al. 2000). Indeed, recent cryo-EM reconstruction of the CrPV IRES bound to 40S subunit reveals that the mRNA region proposed to mimic codon-anticodon interaction at the P site does not overlap with the canonical P-site region (C.M.T. Spahn, personal communication) and thus is "out of reach" for Ede.

The crystal structure *T. thermophilus* 30S subunits bound with Ede (Pioletti et al., 2001) revealed the binding site for this antibiotic to be located between the P and E sites (Figure 1A). Binding of Ede was shown to induce base pair formation between C795 in h24a and G693 in h23b (Figure 1B; *Escherichia coli* numbering used throughout). Whether Ede inhibits P-site binding directly via sterical clashes or whether the inhibition is indirect, perhaps through conformational changes within h23/h24, which in turn could effect positioning of mRNA, remains unclear.

Although pactamycin (Pct) was extensively studied during the 1960–70s and classified as an initiation inhibitor, the mechanism of action of this antibiotic is not clearly defined. The Pct situation in bacteria is referred to in a later review of this literature: Pct "might prevent functional binding of aminoacyl-tRNA into the ribosomal A site. However, this conclusion is, at best, tentative in the absence of more direct evidence" (Gale et al., 1981). In *E. coli* ribosomes, Pct was shown to protect universally conserved bases G693 and C795 of the 16S rRNA from chemical modification (Egebjerg and Garrett, 1991; Woodcock et al., 1991), the same protections found for Ede (Woodcock et al., 1991) and a tRNA bound at the ribosomal P site (Moazed and Noller, 1990). Consistently, Pct-resistant mutants of the archaeon *Halobacterium halobium* were identified at corresponding positions, namely A694G, C795U, and C796U (Mankin, 1997). More recently, the crystal structure of the *T. thermophilus* 30S subunit in complex with Pct was resolved to 3.4 Å (Figure 1A; Brodersen et al., 2000). The single binding site determined on the 30S subunit was in excellent agreement with the biochemical data; the two distal rings of Pct stack upon each other and G693 at the tip of h23b of the 16S rRNA, while the central ring interacts with C795 and C796 in h24a (Figure 1C; Brodersen et al., 2000). Thus, the Pct binding site corresponds remarkably well with the base pair induced when Ede binds to the 30S subunit (compare Figures 1B and 1C).

With the benefit of the crystal structures for the 30S subunit showing a P-site bound tRNA mimic (Carter et al., 2000) and 70S ribosome with three tRNAs bound (Yusupov et al., 2001), it is now clear that Pct binds in the E site and not in the P site as had been thought previously. Furthermore, comparison with these structures suggested that Pct binds in the path of the mRNA such that two of the ring moieties of Pct mimic the last two nucleotides of the E site codon (Brodersen et al., 2000). In doing so, the last position of the E site codon of the mRNA would be displaced by over 12 Å, leading to the suggestion that the inhibition of Pct might be associated with the movement of the mRNA-$tRNA_2$ complex through the ribosome, i.e., translocation.

Here, we have used an in vitro system under near in

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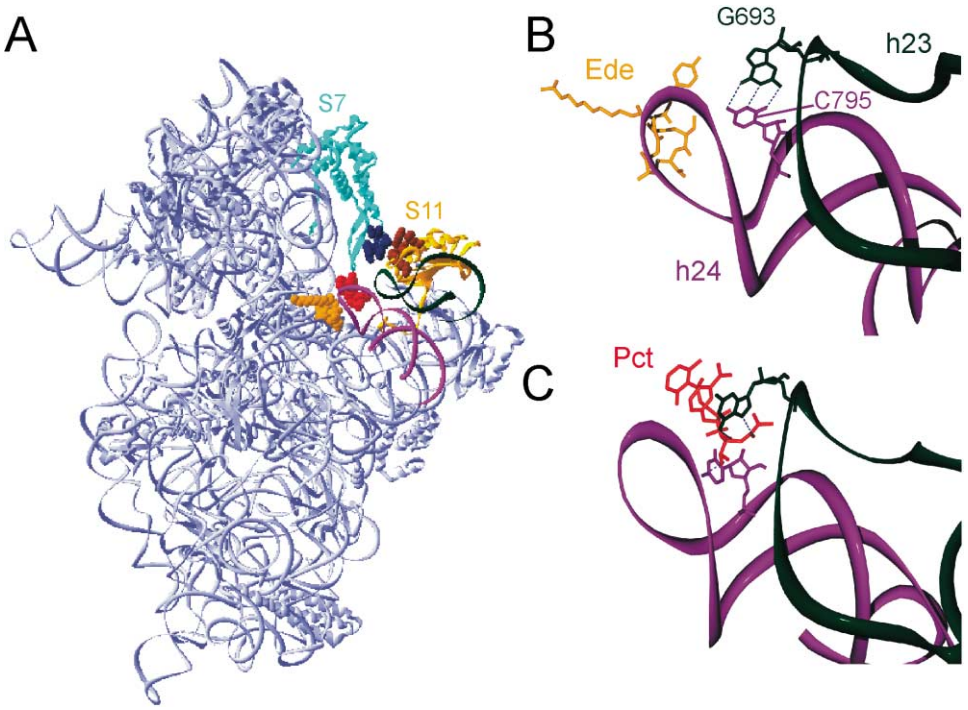

Figure 1. The Binding Site of Edeine and Pactamycin on the 30S Subunit

(A) Relative binding positions of edeine (dark yellow) and pactamycin (red) on the *T. thermophilus* 30S subunit, with the tip of h23 (dark green) and h24 (purple) of the 16S rRNA and the ribosomal protein S7 (cyan) and S11 (yellow). Residues that constitute the interaction between S7 (dark blue) and S11 (brown) are shown in spacefill representation.

(B) Close-up view showing the hydrogen bond formed between residue G693 of h23 and C795 of h24 upon binding of edeine (yellow) to the 30S subunit (pdb 1hnx; Brodersen et al., 2000).

(C) Close-up view of the pactamycin binding site, illustrating the hydrogen bond interactions between G693 and C795 and pactamycin (red) (pdb 1i95; Pioletti et al., 2001). Coloring of helices and bases as in (B).

vivo buffer conditions to systematically analyze the effect of Ede and Pct on the steps of translation initiation and elongation on *E. coli* ribosomes. We find that the pleiotropic inhibitory effects of Ede could be reduced to two basic effects, viz. preventing codon-anticodon interaction at the P site and the induction of translational misreading at the A site. Pct could relieve the P-site effects of Ede and was shown to block the translocation of certain acylated-tRNAs from A to P site completely ($tRNA^{Val}$, $tRNA^{Lys}$), whereas acylated-$tRNA^{Phe}$ remained unaffected. In summary, our results suggest that tRNA binding at the P site is dictated by the conformational state of the universally conserved residues G693 and C795 of the 16S rRNA.

Results

The Half-Inhibition Concentration of Edeine and Pactamycin in a Coupled Transcription-Translation System

The most physiological in vitro system for protein synthesis currently available is the coupled-transcription/translation system. We have utilized this system for the expression of the green fluorescent protein (GFP) gene in order to define the concentration window of Ede and Pct for all subsequent experiments. In Figure 2A, it can be seen that half-inhibitory concentrations (IC_{50}) of Ede and Pct were ~8 μM and ~3 μM, respectively, and that 80% inhibition was seen at concentrations >12.5 μM.

The synthesized GFP was measured both in SDS and native gels, the former assessing the total amount of synthesized protein, whereas the latter enabled the determination of the fluorescence in the gel and thus reflects the fully folded, active fraction of GFP. The expectation is that a drug inducing misincorporations will reduce the active fraction more strongly than the total synthesis, so that the ratio (active fraction)/(total synthesis) will decrease with increasing drug concentration. In contrast, if a drug does not affect the decoding accuracy, the active fraction and total synthesis will decrease proportionally and accordingly the ratio (active fraction)/(total synthesis) will not change with increasing drug concentration. This expectation was verified with streptomycin, the classical inducer of misreading, and tetracycline, which does not affect the decoding accuracy (W.S. and K.H.N., unpublished data). Therefore, this method provides a measure of the overall misincorporation, a feature of the system that is exploited here for the first time. Expression of GFP was measured in the transcription-translation in the presence of increasing concentrations of edeine and pactamycin (Figure 3). The total amount of GFP produced was determined by SDS-PAGE (Figure 3A) and the active GFP fraction by measuring the fluorescence of the GFP protein on native gels

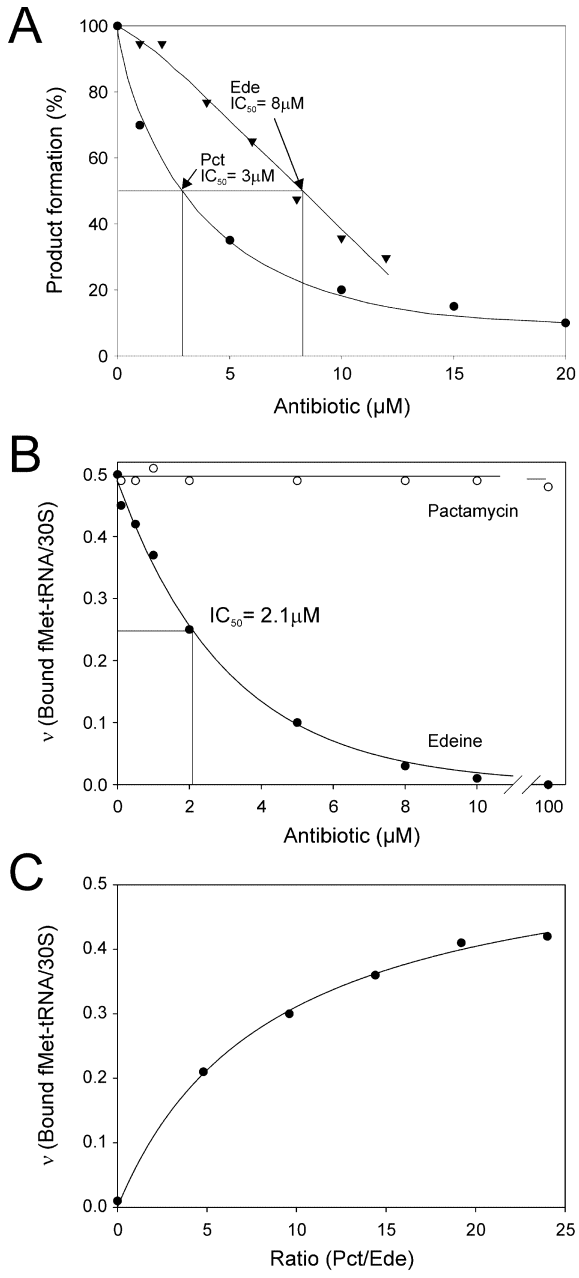

Figure 2. Effect of Edeine and Pactamycin on Coupled Transcription-Translation and 30S Complex Formation

(A) Coupled in vitro transcription-translation was performed in the presence of increasing concentrations of edeine (▼) and pactamycin (●). The relative amount of GFP protein product formed is given as percentages, where 100% is arbitrarily assigned as the product formed in the absence of antibiotic. Experimental details and calculations for translational fidelity are given in Experimental Procedures.

(B) Binding of f[^{3}H]Met-tRNA to MF-mRNA programmed 30S subunits, in the presence of increasing concentrations (0–100 μM) of edeine (●) and pactamycin (○). Binding is presented as ν, which is equivalent to the pmol of tRNA bound per pmol of 30S subunit.

(C) Binding of f[^{3}H]Met-tRNA to MV-mRNA programmed 30S subunits in the presence of increasing ratios of pactamycin (0–200 μM) over edeine (8 μM) to a maximum of 24:1.

(Figure 3B). As described above, the ratio of the active GFP to the total GFP provides a measure of the translational fidelity of the system. As seen in Figure 3C, the ratio of active to total GFP protein did not change with increasing Pct concentrations, demonstrating that Pct does not influence the translational fidelity of the system. In contrast, with increasing Ede concentrations, there was dramatic decrease in the ratio of active GFP protein to total protein, shown in Figure 3C as translational fidelity (solid line with triangle symbols). It follows that Ede is a strong inducer of misincorporations.

The Edeine-Dependent Inhibition of tRNA Binding to 30S Subunits and 70S Ribosomes Is Relieved by Increasing Concentrations of Pactamycin

The initiation phase of translation in bacteria operates predominantly through a preinitiation complex, consisting of fMet-tRNA bound to a programmed 30S subunit with the aid of initiation factors (IFs), which then associates with the 50S subunit to form the 70S-initiation complex (or P_i complex) bearing fMet-tRNA at the P site (reviewed by Gualerzi et al., 2000). The effect of increasing concentrations of Ede and Pct on preinitiation complex formation was analyzed by measuring the binding of f[^{3}H]Met-tRNA to MV-mRNA programmed 30S subunits. As seen in Figure 2B, any concentration of Ede over 8–10 μM completely abolished fMet-tRNA binding (with an IC_{50} of of 2 μM), whereas Pct had no effect, even at excessive concentrations (100 μM). These experiments were repeated in the presence of equimolar or excess amounts of IFs (enzymatic binding) using both Shine-Dalgarno (SD)- and non-SD-containing MV-mRNAs with similar results (data not shown). Furthermore, fMet-tRNA bound in the presence of Pct was puromycin reactive upon addition of 50S subunits regardless of whether the binding was enzymatic or not, i.e., ±IFs (data not shown). This suggests that Pct does not alter the orientation of the P-site tRNA such that formation of the first peptide bond is prevented.

Because the nucleotides of the C795-G693 base pair formed upon Ede binding to the 30S subunit constitute the major component for Pct interaction with 16S rRNA, we reasoned that the addition of Pct might disrupt this base pair and restore binding of fMet-tRNA to 30S subunits. To test this, we reanalyzed fMet-tRNA binding to 30S subunits in the presence of 8 μM Ede, i.e., a concentration where tRNA binding was completely abolished (see Figure 2B), but now with increasing concentrations of Pct. Indeed, we found that addition of Pct in excess restored fMet-tRNA binding to 30S subunits, such that a 25-fold excess of Pct (200 μM) over Ede (8 μM) was sufficient to almost completely restore binding to the level observed in the absence of any antibiotic (Figure 2C).

In order to address the mRNA dependence of Ede inhibition, we compared binding of fMet-tRNA to nonprogrammed and mRNA-programmed 70S ribosomes. This experiment could not be performed with 30S subunits, since there is insignificant binding of tRNAs to nonprogrammed 30S subunits (Gnirke and Nierhaus, 1986). We found that with increasing concentrations of

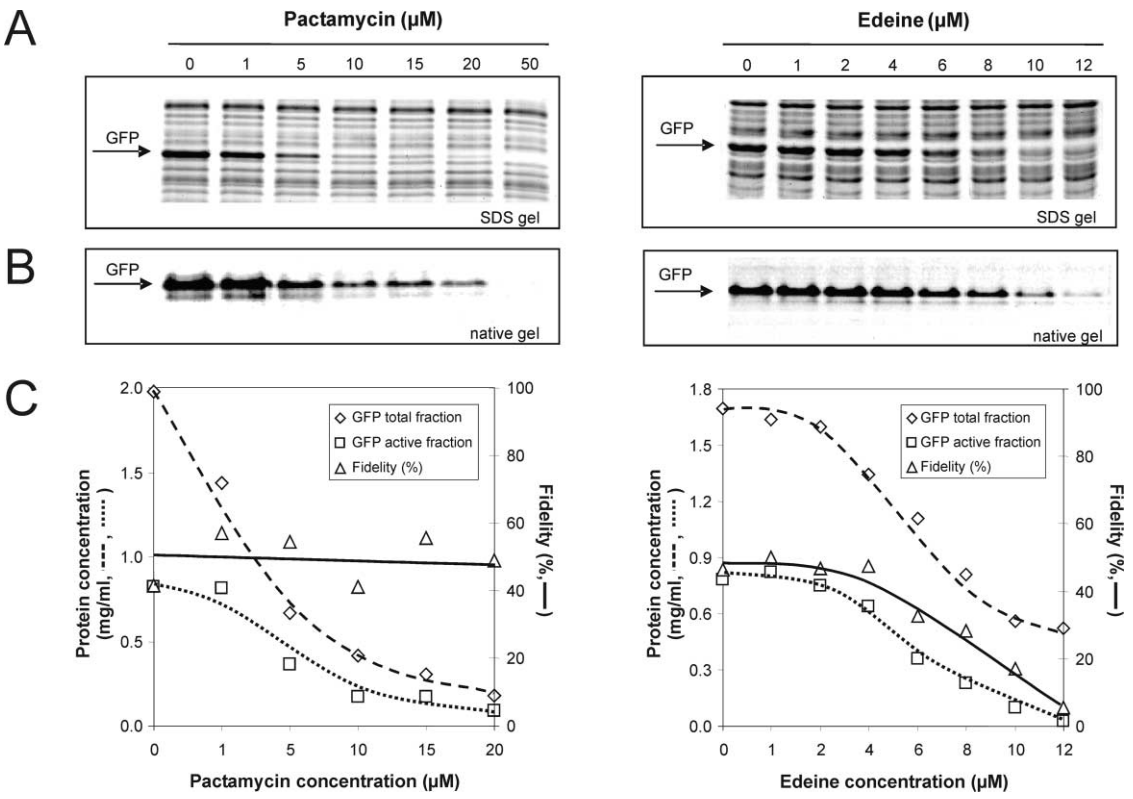

Figure 3. Expression of GFP in a Transcription-Translation Assay in the Presence of Increasing Concentrations of Edeine and Pactamycin

The total amount of GFP produced was determined by SDS-PAGE (A) and the active GFP fraction by measuring the fluorescence of the GFP protein on native gels (B). The ratio of the active GFP to the total GFP provides a measure of the translational fidelity of the system. As seen in (C), the amount of active (□) to total GFP (◇) protein did not change with increasing pactamycin concentrations, demonstrating that pactamycin does not influence the translational fidelity (△) of the system. In contrast, with increasing edeine concentrations, there was dramatic decrease in the ratio of active GFP protein to total protein, shown in (C) as translational fidelity (solid line with triangle symbols).

Ede, programmed fMet-tRNA binding was significantly affected, eventually being reduced to nonprogrammed binding levels at concentrations of 20 μM or higher (Figure 4A). The IC_{50} of <2 μM was similar to that observed in Figure 2B for fMet-tRNA binding to 30S subunits. At higher (nonphysiological) Ede concentrations (20–100 μM), nonprogrammed fMet-tRNA binding was also affected (50%). When the same experiments were repeated with Pct in place of Ede, we observed little or no inhibition on fMet-tRNA binding to 70S ribosomes in the presence or absence of the MF-mRNA (data not shown).

Similarly, AcPhe-tRNA binding to MF-mRNA programmed 70S ribosomes was not inhibited by Pct, whereas Ede reduced the binding to the level seen with nonprogrammed 70S ribosomes, but abolished programmed binding above 15 μM (Figure 4B), with an IC_{50} of 5 μM (data not shown). Again, we observed that in the presence of 30-fold excess of Pct over Ede (15 μM), inhibition of AcPhe-tRNA binding to 70S ribosomes could be fully restored (inset to Figure 3B). Finally, because binding of tRNA to the P site of 70S ribosomes induces protection patterns similar to those of Ede (Moazed and Noller, 1987; Woodcock et al., 1991), we tested the influence of Ede when added following formation of the programmed 30S•fMet-tRNA complex. Even high concentrations of Ede (100 μM) had no inhibitory effect (data not shown), suggesting that stably bound P-site tRNA is immune to the effects of Ede, as observed by Szer and colleague at lower Ede concentrations (Szer and Kurylo-Borowska, 1970).

Pactamycin Allows A-Site tRNA Binding but Inhibits Translocation in a Manner Dependent on the tRNA-mRNA Complex

Having found no inhibitory effect of Pct up to the point of P_i-state formation, we were interested in analyzing the subsequent steps; namely, A-site tRNA binding and the first translocation reaction. No inhibition of A-site binding was observed in the presence of up to 200 μM Pct, irrespective of the various N-acetyl-aminoacyl-tRNA species tested (Figure 5A, black bars in sets 1–3), nor with up to 100 μM Ede (data not shown). Similarly, Pct did not inhibit enzymatic binding of aminoacyl-tRNAs, Phe-tRNA, or Val-tRNA (Figure 5A, black bars in sets 4 and 5) to the A site, when fMet-tRNA was prebound at the P site. In the latter experiment, the step of peptide bond formation is included in the experiment, whereas the former mimics the situation following peptide bond formation but before translocation. In contrast with Ede and Pct, our control antibiotic tetracycline (Tet), a well-known A-site inhibitor (Geigenmüller and Nierhaus, 1986), strongly prevented tRNA binding to the A

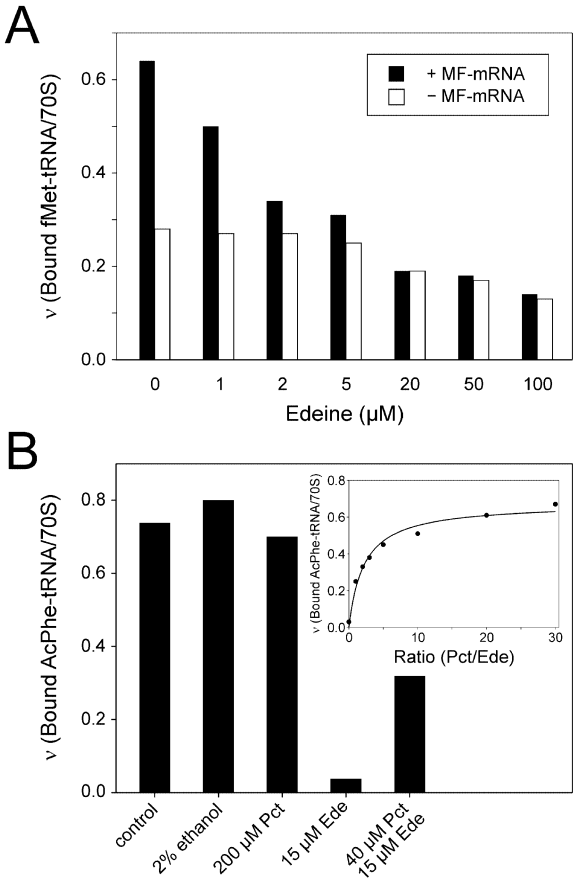

Figure 4. Effect of Edeine and Pactamycin on 70S Complex Formation

(A) Binding of f[^{3}H]Met-tRNA to 70S ribosomes was measured in the presence (black bars) or absence (white bars) of MF-mRNA and with increasing concentrations of edeine (0–100 μM). Binding is presented as ν, which is equivalent to the pmol of tRNA bound per pmol of 70S.

(B) Binding of Ac[^{14}C]Phe-tRNA to MF-mRNA programmed 70S ribosomes was performed in the absence of antibiotic (control, 2% ethanol) or in the presence of antibiotics (all in 2% ethanol) as indicated. The inset in (B) shows the binding of Ac[^{14}C]Phe-tRNA to MF-mRNA programmed 70S ribosomes with an increasing amounts of pactamycin over edeine (15 μM) up to 30-fold.

site at 100 μM for all tRNA-mRNA combinations tested (shaded bars).

Next, translocation of these complexes was measured in the presence and absence of Pct (Figure 5B). Here, we observed strong inhibition of translocation in the presence of Pct concentrations above 5 μM using MV-mRNA with $tRNA_f^{Met}$ in the P site and AcVal-tRNA in the A site, with an IC_{50} <1 μM. However, to our surprise, little or no translocation inhibition by Pct (at most 15% inhibition at 200 μM Pct) was observed when MF-mRNA-programmed ribosomes were used, bearing either deacylated $tRNA_f^{Met}$ at the P site and NAcPhe-tRNA at the A site (Figure 5B) or fMet-tRNA at the P site and Phe-tRNA at the A site (data not shown).

To ensure that the translocation inhibition was not specific for the MV-mRNA/Val-tRNA combination, we repeated the experiment using poly(A)-programmed 70S ribosomes with deacylated $tRNA^{Lys}$ at the P site and AcLys-tRNA at the A site. As shown in Figure 5C, Pct inhibits translocation of this complex with an IC_{50} of <0.25 μM. We note that the incubation time for each translocation assay was 10 min, i.e., much longer than the 0.1 s required for a total elongation cycle in our poly(U) system. This means that even under conditions where ribosomes are given ample time to translocate, Pct still effectively blocks this reaction, demonstrating the general potency of this translocation inhibition.

Pactamycin Prevents Entry of the tRNA-mRNA Complex into the E Site

To conclusively demonstrate that pactamycin prevents movement of the tRNA-mRNA complex into the E site, we utilized MVF-mRNA, which enables successive binding of three tRNAs to the ribosome to be monitored. First, binding of [^{32}P] $tRNA_f^{Met}$ to the P site and Ac[^{14}C]Val-tRNA to the A site to form a pretranslocation state (PRE-1) was performed in the absence and presence of Pct, which was followed by addition of EF-G to transfer ribosomes into the posttranslocation state (POST-1). As expected, the binding of $tRNA_f^{Met}$ (Figure 6, blue bars) and AcVal-tRNA (red bars) in both PRE-1 and POST-1 states were identical in the absence of Pct (Figure 6A). Almost all the AcVal-tRNA (ν = 0.58 pmols bound per pmol 70S) is translocated to the P site and is therefore reactive with puromycin (ν = 0.55 pmols AcVal-puromycin formed per pmol 70S). However, in the presence of Pct, AcVal-puromycin formation was almost completely inhibited (ν = 0.04; Figure 6B), indicating that the A site was still occupied by the AcVal-tRNA. In the next step, the availability of the A site was verified by addition of the ternary complex EF-Tu•GTP•[^{3}H]Phe-tRNA, which is cognate for the A-site UUC codon. We observed good binding of Phe-tRNA to the A site in the absence of Pct but very little binding in the presence of Pct (compare green bars in Figures 6A [ν = 0.52] and 6B [ν = 0.06], respectively). This is consistent with PRE-2 state formation in the absence of Pct and with translocation inhibition by Pct, which freezes the ribosome in the PRE-1 state. Furthermore, the binding of the ternary complex to the A site of the POST-1 state induced release of the E site tRNA (change in ν from 0.80 to 0.15), in accord with the reciprocal linkage proposed to exist between A and E sites (reviewed by Blaha and Nierhaus, 2001).

Pactamycin Severely Inhibits Poly(A)-Dependent Poly(Lys) Synthesis but Not Poly(U)-Dependent Poly(Phe) Synthesis

Since pretranslocational complexes with Phe-tRNA and MF-mRNA exhibited "immunity" to the inhibitory effects of Pct, in contrast to the Lys-tRNA/poly(A) combination, we were interested in determining whether this bias was also reflected in the poly(U)-dependent poly(Phe) and poly(A)-dependent poly(Lys) in vitro translation systems.

To test this, we performed poly(A)-dependent poly (Lys) synthesis in the presence of Pct, Ede, and Tet. Our results demonstrated that Pct was an effective inhibitor of poly(Lys) formation, as was Ede and the control antibiotic Tet (Figure 7A). As expected, Pct did not relieve the inhibition due to Ede, since Pct itself inhibited the reaction. The inset to Figure 7A illustrates the potency of Pct, since an inhibition plateau was reached at con-

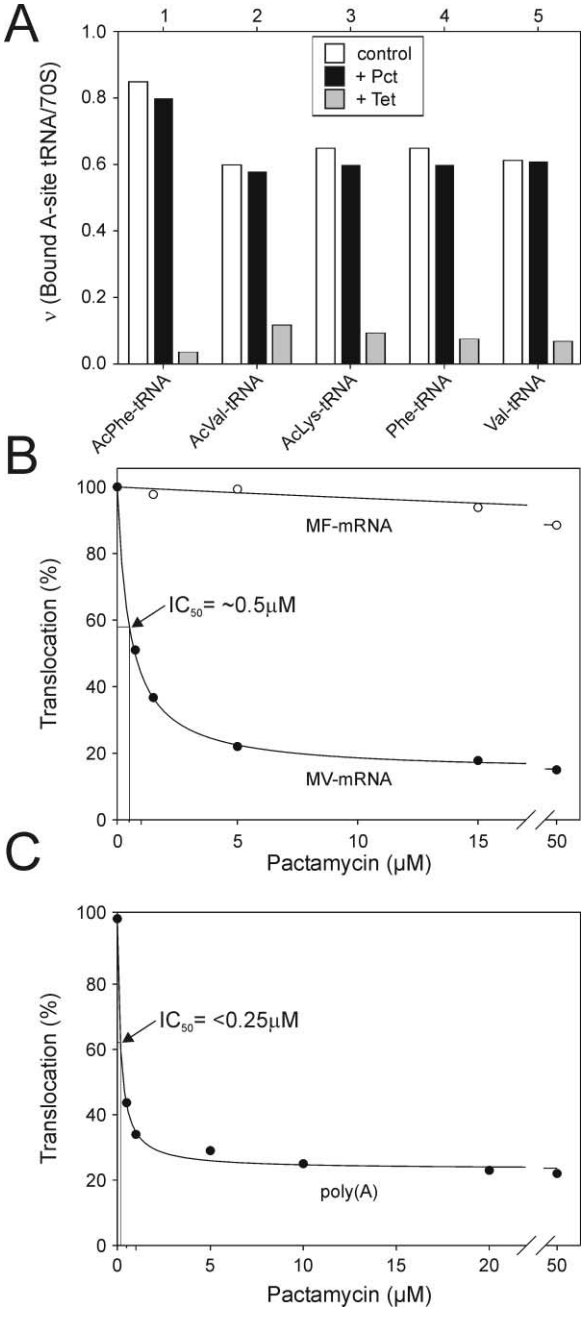

Figure 5. Effect of Pactamycin on A-Site Binding and Translocation

(A) Enzymatic and nonenzymatic A-site binding to various P_i state ribosomal complexes without antibiotic (white bars) or in the presence of 100 μM pactamycin (black bars) or 350 μM tetracycline (gray bars). Binding of AcPhe-tRNA (1), Ac[^{14}C]Val-tRNA (2), or Ac[^{14}C]Lys-tRNA (3) to MF-mRNA (1), MV-mRNA (2), or poly(A) (3) programmed 70S ribosomes with deacylated $tRNA_f^{Met}$ (1, 2) or $tRNA^{Lys}$ (3) bound at the P site. Binding of ternary complexes containing Phe-tRNA (4) or Val-tRNA (5) to MF- (4) or MV-mRNA (5) programmed 70S ribosomes with fMet-tRNA bound at the P site.

(B) EF-G-dependent translocation of AcPhe-tRNA (●) or AcVal-tRNA (○) with deacylated $tRNA_f^{Met}$ at the P site in the presence of increasing pactamycin concentrations (0–50 μM). The corresponding mRNAs are indicated.

(C) EF-G-dependent translocation of poly(A) programmed 70S ribosomes with Ac[^{14}C]Lys-tRNA and $tRNA^{Lys}$ at A and P sites, respectively, in the presence of increasing concentrations of pactamycin (0–50 μM). Translocation of 100% represents conversion of all PRE ribosomes (which for [B] and [C] was equivalent to 60%–80% of the ribosomes, as seen in [A]) into the post state.

centrations nearing 20 μM, and the IC_{50} was determined to be within the range of 3 μM, which was comparable with the IC_{50} for Pct in the coupled system (Figure 2A). We note that Tet was a relatively poor inhibitor of the poly(Lys) synthesis, requiring drug concentrations of more than 300 μM to obtain near complete inhibition.

However, when the poly(U)-dependent poly(Phe) system was employed, no inhibition of poly(Phe) synthesis by Pct was observed, even at very high concentrations (up to 200 μM). In fact, Pct actually enhanced the extent of translation up to 2-fold over the control reaction after 60 min (Figure 7B). In contrast, Ede, which was shown previously to inhibit AcPhe-tRNA binding to 70S ribosomes (Figure 4B), severely inhibited poly(Phe) formation (Figures 7B and 7C); at 80 μM Ede, over 80% inhibition was observed (Figure 7C, lane 3), with an IC_{50} of 10 μM (data not shown). The effect of Ede was much more dramatic than that of Tet, which caused 65% inhibition of poly(Phe) synthesis at concentrations of 350 μM (Figure 7C, lane 7), as observed previously (Hausner et al., 1988). Consistent with our binding results, we were able to show that Pct could also relieve the in vitro translation inhibition caused by Ede, such that addition of 2.5-fold excess of Pct (200 μM) over Ede (80 μM) restored Phe incorporation to the enhanced level observed in the presence of Pct alone (Figure 7C, lane 4).

Edeine and Pactamycin Effects on Misreading during Poly(Phe) Synthesis

The increase in poly(Phe) synthesis in the presence of Pct (Figure 7B) and the loss of translational fidelity seen in the coupled system in the presence of Ede (Figure 2A) prompted us to investigate the level of misincorporation in the presence of these antibiotics. For a control, we used streptomycin (Str), an antibiotic well known for its strong stimulatory effect on translational misreading (Spahn and Prescott, 1996, and references therein). To monitor the translational error, the poly(Phe) reactions were spiked with radiolabeled leucine (Leu), since the tRNAs encoding this amino acid are near cognate for the Phe codon UUU. The highly optimized poly(U)-dependent poly(Phe) system that we are using can synthesize at rates approaching 20 Phe/(ribosome × min) with an initial rate up to 8 Phe/(ribosome × s) (data not shown) and an accuracy nearing in vivo, i.e., approaching one misincorporation per 1000–3000 amino acids incorporated (Kurland et al., 1990, and references therein). As shown in Figure 7D, our system was characterized by a "background" rate (with 2% ethanol) of misincorporation of ~1 Leu for every 1900 Phe (lane 1). To our surprise and despite the increased rate of Phe incorporation when Pct was present, we did not observe a corresponding increase in the level of translational misreading (Figure 7D, lane 2). In fact, we found that Pct increased the fidelity of translation up to 1/2900. This latter point is even more significant when one considers that poly(Phe) formation in the presence of Pct

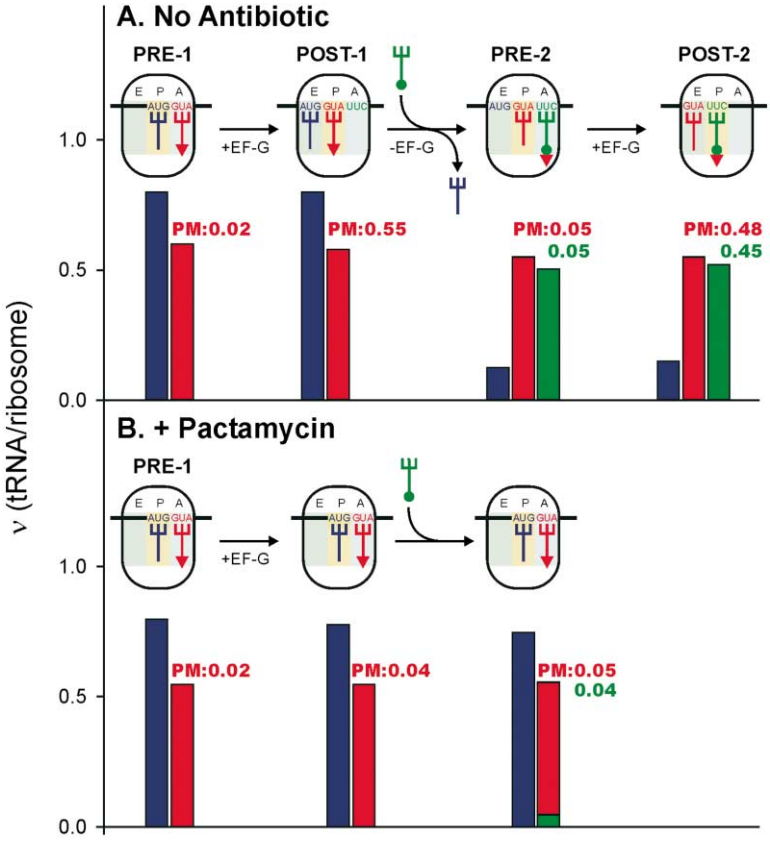

Figure 6. Pactamycin Inhibits Translocation by Preventing tRNA Movement into the E Site

The successive steps of the elongation cycle are monitored in the absence (A) or presence of 10 μM pactamycin (B). The binding of [^{32}P] $tRNA_{\nu}^{Met}$ (blue) to the P site and Ac[^{14}C]Val-tRNA (red) to the A site (PRE-1 formation) was followed by addition of EF-G and GTP to induce translocation (POST-1). EF-G was removed from POST-1 complexes and the ternary complex EF-Tu•GTP•[^{3}H]Phe-tRNA (green) was added to form a new pretranslocational complex (PRE-2), which with readdition of EF-G formed the respective POST-2 complex. In all cases, the binding of the respective tRNAs are indicated with bars of the corresponding color, and the accessibility of the A site to puromycin (PM) is indicated with values adjacent to the bar of the same color. All data are expressed with ν, i.e., pmoles of bound tRNA, or peptidyl-puromycin formed, per pmol ribosome.

is almost twice as high as the control after 60 min (Figure 7B).

In contrast, we found that Ede dramatically increased the level of misincorporation, although it significantly reduced the overall formation of poly(Phe). At 80 μM, Ede induced 1/150 misincorporations (Figure 7D, lane 3). This level of misincorporation remained constant when the Ede concentration ranged from 10 to 100 μM (data not shown), which was within a similar misincorporation range to that of Str (1/160, Figure 7D, lane 5); however, unlike Ede, Str stimulated poly(Phe) synthesis in a similar fashion to Pct (Figure 7C, lane 5). Of special interest to us was that the level of misincorporation in the presence of both Ede (80 μM) and Pct (200 μM) was identical to that in the presence of Ede alone (compare Figure 7D, lanes 3 and 4), yet the poly(Phe) formation was increased, comparable to that in the presence of Pct alone (compare Figure 7C, lanes 2 and 4).

Since Pct seemed to have a beneficial effect in terms of stimulating the rate of poly(Phe) synthesis as well as reducing the errors, we were interested to see whether Pct could also counteract the misreading effects of Str. In the presence of 10 μM Str and 20-fold excess of Pct, the number of misincorporations were reduced by 25%, while the poly(Phe) formation remained unchanged, suggesting that there is a different interplay between Pct and Str than between Pct and Ede. Finally, we note that a combination of Str and Ede exhibited a phenotype similar to Str alone, i.e., Phe formation was stimulated (2.5-fold) but at the loss of fidelity; however, the misincorporation effects of Str and Ede were not additive (data not shown).

Discussion

The Inhibitory Effects of Edeine to Prevent tRNA Binding at the P Site Can Be Attributed to Preventing Codon-Anticodon Interaction

Our in vitro analysis of the mechanism of inhibition of Ede supports the role of Ede as a powerful inhibitor of initiation. We found that Ede strongly inhibited the binding of fMet-tRNA to 30S subunits, with complete inhibition observed at 10 μM and an IC_{50} of 2 μM. However, addition of Ede after formation of the 30S initiation complex had no effect on the binding of fMet-tRNA, suggesting that the binding site of Ede was perturbed or partially occupied and thus the initiation complex is stabilized against the effects of Ede. In Figure 4A, low concentrations of Ede (<10 μM) were shown to reduce tRNA binding to 70S ribosomes to the level of nonprogrammed ribosomes.

The major effects of edeine can be explained by assuming that Ede blocks exclusively codon-anticodon interaction at the P site: since tRNA binding at the P site of 30S subunits occurs strictly in the presence of codon-anticodon interaction (Gnirke and Nierhaus, 1986), this is consistent with the total blockage of tRNA binding to the small subunit observed in the presence of Ede. Likewise, on the 70S subunit, in the absence of mRNA (and therefore, of codon-anticodon interaction), a P-site bound tRNA has lost its contacts with the 30S subunit, instead interaction is made almost exclusively with the 50S subunit of the 70S ribosome (Schäfer et al., 2002). By preventing codon-anticodon interaction, Ede reduces the level of tRNA binding to 70S ribosomes

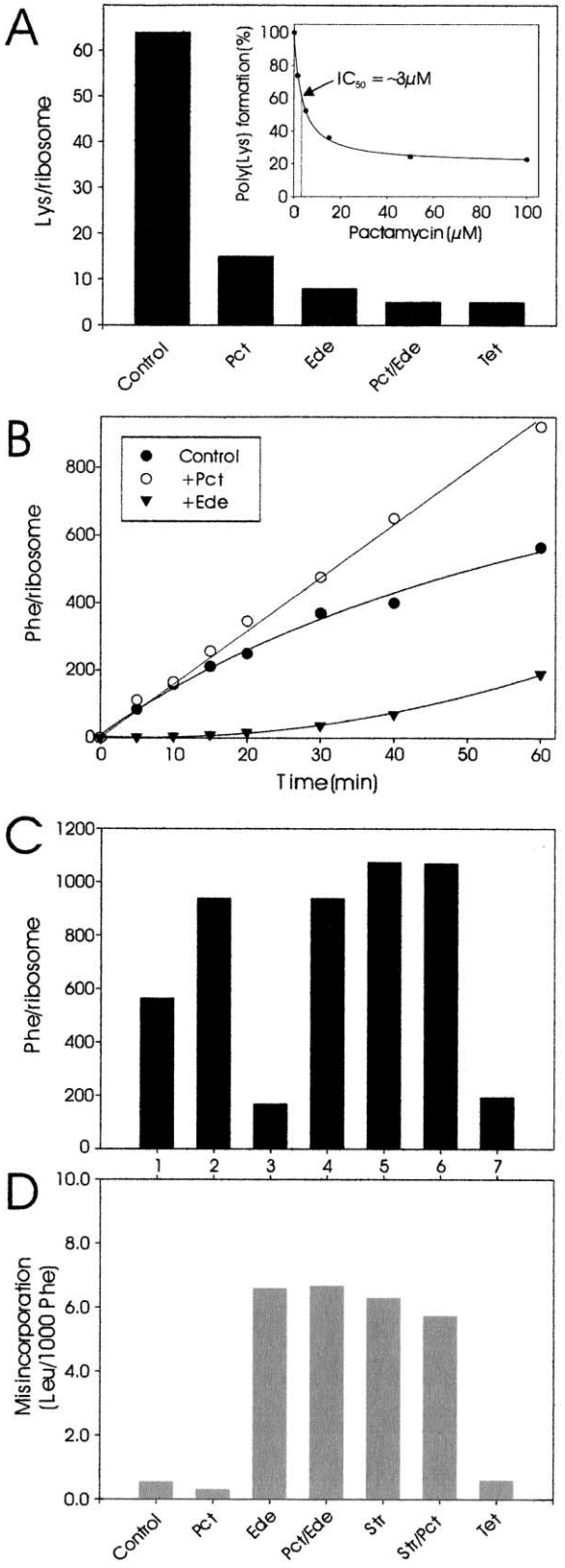

Figure 7. Effect of Edeine and Pactamycin on Poly(Lys) and Poly(Phe) Formation and Misincorporation

(A) Polylysine formation in the absence (control contains 2% ethanol) and presence of the following antibiotics: 50 μM pactamycin (Pct), 50 μM edeine (Ede), 50 μM of both pactamycin and edeine (Pct/Ede), and a control reaction with 300 μM tetracycline (Tet). The insert shows the effect of increasing pactamycin concentrations (0–100 μM) on Poly(Lys) formation, where 100% is equivalent to 60 Lys/ribosome, observed in the absence of antibiotic (control bar). IC_{50} was determined to be in the range of 3 μM. All Lys/ribosome values were determined from duplicate values taken from 60 min time points (see Experimental Procedures). (B) Time plot of poly(U)-dependent poly(Phe) synthesis in the presence of 200 μM pactamycin (○), 80 μM edeine (▼), and in the absence of antibiotic (control [●] with 2% ethanol). (C) Poly(Phe) formation and (D) misincorporation (Leu/1000 Phe), in the absence (control with 2% ethanol, bar 1) and presence of 200 μM pactamycin (Pct, bar 2), 80 μM edeine (Ede, bar 3), both 200 μM pactamycin and 80 μM edeine (Pct/Ede, bar 4), 10 μM streptomycin (Str, bar 5), both 200 μM pactamycin and 10 μM streptomycin (Str/Pct, bar 6), and 350 μM tetracycline (Tet, bar 7). Since the rate of poly(Phe) formation was not linear (as seen in [B]), the values in (C) and (D) are presented only for the 60 min time point.

to that observed in the presence of nonprogrammed ribosomes. At Ede concentrations above 15 μM, the binding of fMet-tRNA to programmed or nonprogrammed 70S ribosomes is marginally reduced, whereas the P-site binding of the artificial peptidyl-tRNA mimic AcPhe-tRNA was completely blocked, providing some indication that these tRNAs bind differently on the ribosome. However, once the tRNA-mRNA interactions with the ribosome are established, particularly the interaction of the mRNA in the E site with the tips of h23/h24 (Brodersen et al., 2000), Ede cannot undo them. In this respect, it is interesting to mention that Ede could remove the P-site tRNA from the 30S, when the mRNA was replaced with a single triplet codon (Szer and Kurylo-Borowska, 1970), since in this case, no interaction between mRNA and h23/h24 is possible.

We were surprised to find that Ede significantly enhanced misincorporation and astonished to find that levels paralleled those of the classic misreading antibiotic Str. This finding is of particular interest since, to date, misreading antibiotics have been discovered that bind either to the decoding center of the A site, like aminoglycosides, or within the vicinity of h27, such as Str, but not in proximity to the E site. It was recently reported that chloramphenicol, as well as the oxazolidinone class of antibiotics, which bind within the PTF center of the large subunit, induce miscoding events, such as frameshifting and nonsense suppression (Thompson et al., 2002), supporting the notion that certain antibiotics can exert long-range effects on the fidelity of translation. Indeed, a rational explanation can be put forward for Ede-induced misreading, since it has been demonstrated that the presence of the E-site tRNA enhances the fidelity of decoding at the A site (reviewed by Blaha and Nierhaus, 2001): the G693-C795 base pair induced by Ede binding disrupts the path of the mRNA through the E site (Pioletti et al., 2001); therefore, it is conceivable that the allosteric effect that the E-site tRNA normally conveys to fidelity of A-site decoding is impaired. In this case, the poststate ribosome in the presence of Ede may not have fully converted from the closed to the open 30S conformation (Ogle et al., 2003), thus increasing the probability that near-cognate aa-tRNAs are accepted at the A site, in a situation where they would normally be rejected. The idea that occupation of the E site affects

A-site fidelity has gained support from a recent observation: mutant ribosomes where the S7-S11 interface at the E site is disrupted at the positions indicated in Figure 1A led to a loss of translational fidelity (misreading, frameshifting, and nonsense suppression; Robert and Brakier-Gingras, 2003).

Pactamycin as a Universal Translocation Inhibitor

Pct has been classified for historical reasons as an initiation inhibitor, a classification propagated through more recent publications. Although we cannot explicitly rule out additional subtle inhibitory effects of Pct during the initiation phase, our results favor a role of Pct as an inhibitor of the translocation reaction. This conclusion is based on the observation that Pct does not inhibit (1) enzymatic or nonenzymatic binding of fMet-tRNA to programmed 30S subunits (Figure 2B), (2) association of this preinitiation complex with the 50S subunit, nor (3) the reactivity of fMet-tRNA with puromycin within the Pi complex. In fact, we could find no significant inhibitory effect up to and including the formation of the pretranslocation complex, i.e., a programmed ribosome containing tRNAs at the A and P site. However, the next step, translocation, revealed itself to be the target of Pct. We have shown that the presence of >5 μM Pct was sufficient to effectively abolish translocation of two different mRNA-tRNA combinations (Figures 5B and 5C). We also found that the IC_{50} of Pct for inhibition of poly(A)-dependent poly(Lys) synthesis was within the 3 μM range (Figure 7A).

We believe an IC_{50} of <3 μM to be within the physiological range of this antibiotic, since the growth of the *E. coli* strain MRE600 is completely inhibited at concentrations over 5 μM (D.N.W. and K.H.N., unpublished data), and the inhibition seen with Pct in the coupled transcription-translation system was also similar ($IC_{50} = 3$ μM from Figure 2A). Additionally, inhibition of growth of the archaeon *H. Halobium* was also shown to be complete at 10 μM (Mankin, 1997). We note that only 40%–50% inhibition of translocation has been obtained at higher concentrations (50 μM) of well-documented translocation inhibitors, such as the aminoglycosides neomycin and hygromycin B (Hausner et al., 1988), demonstrating that Pct can be classified as a potent inhibitor of translocation.

Based on the binding position of Pct on the 30S subunit, Pct was proposed to (1) reduce the flexibility of the 30S subunit by locking h23 and h24 together and (2) obstruct the mRNA, such that the E site codon is displaced by over 12 Å (Brodersen et al., 2000). Both predictions can be reconciled by the translocation inhibition effect of Pct: (1) cryo-EM reconstructions suggest a ratchet motion during translocation where the ribosome undergoes large-scale conformational changes of the head of 30S subunit relative to the platform region where Pct binds (Valle et al., 2003). If Pct prevents this movement, a blockage of translocation will occur. (2) Translocation requires movement of the mRNA-$tRNA_2$ complex from the A and P site into the P and E sites, respectively. Therefore, displacement of the path of the mRNA through the E site may prevent entry into the E site and thus block translocation. However, what is a surprise and not so easily explainable is why Pct does not inhibit translocation of complexes with peptidyl-$tRNA^{Phe}$. In the absence of high-resolution structures for the various pre- and posttranslocational states, we can only assume that certain tRNA-mRNA combinations interact with the ribosome stronger than others or that these interactions are simply not available to particular tRNAs or mRNAs. Further studies are underway to address these issues.

Since the binding site for Pct on the 30S subunit consists only of universally conserved residues of the 16S rRNA, it could be expected that the inhibition mechanism of Pct would also be identical across the three phylogenetic kingdoms. In this regard, we note that numerous studies in eukaryotes are compatible with Pct inhibition of the first translocation step. Specifically, these studies observed large accumulation of dipeptide on the ribosome in the presence of Pct (Cheung et al., 1973; Kappen and Goldberg, 1976). Cheung and coworkers observed both an accumulation of Met-Met and Met-Val dipeptides in the presence of 1 μM Pct using a poly(AUG) translation system, explaining the latter dipeptide as due to the presence of contaminating endogenous hemoglobin mRNA. In these experiments virtually no tripeptide or higher oligopeptides were detected. In another study, performed in the presence of stoichiometric amounts of IFs, no inhibition of initiation in the presence of Pct was observed using an globin-synthesizing rabbit reticulocyte lysate in vitro translation system (Kappen and Goldberg, 1976). Instead, an accumulation of the dipeptide Met-Val was observed in the P site of the ribosome; however, in contrast with our findings, the dipeptide was reported to be puromycin reactive, leading to the conclusion that the inhibition was posttranslocational.

The Antagonistic Interplay between Pactamycin and Edeine Highlights the Importance of the G693:C795 Base Pair for P-Site tRNA Binding

Many antibiotics compete for similar binding sites within the decoding or PTF center, an example of the latter being chloramphenicol and puromycin, but to our knowledge, such a dramatic antagonistic relationship between two antibiotics on the ribosome has never been reported previously. Furthermore, we believe the interplay between Pct and Ede sets a nice example of how antibiotic-subunit structures can provide a tool to dissect the mode of antibiotic action by biochemical experimentation. Thus, from the crystal structure of Ede and Pct bound to the 30S subunit, we predicted that Pct binding might reverse the effects of Ede by breaking the G693:C795 base pair. We demonstrated that the observed interplay between Pct and Ede is consistent with this prediction and is reproducible under three distinct situations, i.e., Pct relieves the Ede induced inhibition of (1) fMet-tRNA binding to 30S, (2) AcPhe-tRNA binding to 70S, as well as (3) a poly(U)-dependent poly(Phe) in vitro translation system. We do not believe Pct relieves the inhibition by removing Ede from the ribosome, since we find that poly(U)-dependent poly(Phe) in the presence of both Pct and Ede exhibits features attributable to both antibiotics, namely, an increased rate of synthesis (due to Pct) along with a high rate of misincorporation observed in the presence of Ede. We reason that the reversal of inhibition by Pct

binding results from breaking the G693:C795 base pair formed upon Ede binding. The implication being that tRNA binding to the P site of the 30S and 70S requires a non-base-paired conformation of G693 and C795, but upon binding to the P site the base pair is formed again. This is supported by the observation that binding of tRNA to the P site of 70S ribosomes led to protection of both bases (Moazed and Noller, 1990). Therefore, we propose that Ede, by inducing the G693:C795 base pair, mimics a ribosome state where the P site tRNA is already bound and that Pct restores the conformation to the unbound state. However, although in the presence of Pct P site tRNA binding can occur, the lack of the G693:C795 base pair and/or the presence of Pct obviously result in preventing the later translocation event.

A provocative proposal has been made that low molecular weight molecules, such as small RNAs or antibiotic progenitors, may have been present in the primordial soup and coevolved with the modern ribosome, originally serving a regulator function (Davies, 1990). Support for this proposal was provided to some extent by the finding that aminoglycoside antibiotics were found that inhibited two ancient and distinct ribozymes, namely the ribosome and group I introns (von Ahsen et al., 1991). Our finding that Ede and Pct work antagonistically to influence initiation complex formation certainly demonstrates the potential existing for such small molecule regulation.

Experimental Procedures

Preparation of Purified Components for In Vitro Assays

All experiments utilized reassociated 70S ribosomes or 30S subunits, prepared according to Blaha et al. (2000). MF-mRNA, encoding Met-Phe [sequence: GGG$(A_4G)_3$AAAAUGUUC$(A_4G)_3$AAAU] (Triana-Alonso et al., 1995) and MV(F)-mRNA with **SD** [GGGAAAA **GGAGGU**CACAUAUGGUA*UUC*$(A_4G)_3$AAAU] and without SD sequence [GGG$(A_4G)_3$AAAAUGGUA*UUC*$(A_4G)_3$AAAU], encoding Met-Val-(*Phe*), were prepared according to Schäfer et al. (2002). Poly(U) and poly(A) were purchased from Roche. Poly(U) was fractionated, and only fractions of 50 ± 25 bases were used (Schäfer et al., 2002). EF-Tu and EF-G with C-terminal His-tags were isolated from *E. coli*, as described previously for EF-Tu (Boon et al., 1992). Initiation factors IF1, IF2, and IF3 were prepared as described by Pawlik et al. (1981). Crude or specific tRNAs were purchased from Sigma and charged according to Rheinberger et al. (1988).

Coupled Transcription-Translation Assay

All coupled transcription-translation experiments were performed using the *E.coli* lysate-based system (RTS 100 *E. coli* HY Kit from Roche) for the expression of the green fluorescence protein (GFP) type cyc3 (Crameri et al., 1996) from the template plasmid pIVEX2.2-GFPcyc (kindly provided by Roche). Reactions were executed as per manufacturer's instructions, with the exception that the final reaction volume was reduced from 50 μl to 10 μl and that the relevant antibiotics were included. In brief, the lyophilized components supplied in RTS 100 kit were reconstituted and mixed. Each reaction contained 2.4 μl *E. coli* lysate, 2 μl reaction mix, 2.4 μl amino acids, 0.2 μl methionine, 1 μl reconstitution buffer, 1 μl pIVEX2.2-GFPcyc3 (0.1 μg/μl), and 0.5 μl antibiotic or control buffer. Reactions were incubated for 5 hr at 30°C with shaking (900 rpm) in the RTS ProteoMaster Instrument (Roche).

Quantification of Translation Fidelity

From each 10 μl reaction, 1.5 μl was analyzed on a 15% PAGE, under denaturing conditions with 2% SDS according to (Laemmli and Favre, 1973) or after a maturation period of about 16 hr at 4°C under native conditions (Maniatis et al., 1982). On each gel, reference lanes were included where a known amount of pure and active GFP protein (Roche) was loaded. The denaturing gels were run for 3 hr at 150 V, which enabled good separation of the GFP protein band from neighboring bands. The SDS-PAGE gels were scanned and the GFP bands quantitated and the total amount of GFP in each lane was determined by comparison with the reference GFP protein. On average, ~1.7 mg/ml were produced in 5 hr in the absence of antibiotic.

The active GFP present in each reaction sample was calculated by measuring the fluorescence 430–580 nm of the GFP proteins in the native PAGE. The reference GFP were arbitrarily assigned as 100%, and the relative activity of the newly translated GFP protein was calculated. On average, the activity of the GFP from the coupled in vitro system performed in the absence of antibiotic was 50% of that of the reference GFP.

To calculate the translational fidelity, the amount of active GFP protein was compared with the total amount of GFP protein produced at each antibiotic concentration. A decrease in translational fidelity will result in a corresponding decrease in active protein, since misreading events will result in the incorporation of incorrect amino acids, eventually leading to loss of activity.

Preparation of Defined Ribosomal Complexes

All complexes were prepared under identical ionic conditions, namely, 20 mM HEPES-KOH (pH 7.6), 4.5 mM magnesium acetate, 150 mM ammonium acetate, 2 mM spermidine, 0.05 mM spermine, and 4 mM β-mercaptoethanol, and were kept constant throughout all the steps of complex formation. Reassociated 70S ribosomes or 30S subunits were used at final concentration of 0.3 μM and were incubated in the presence of the appropriate mRNA (2.0 μM) and the charged tRNA (0.5 μM) at 37°C for 15 min (as described by Blaha et al., 2000), unless otherwise indicated. TRNA binding was assessed, and the puromycin reaction was performed as described by Blaha et al. (2000). All single determinations were performed in duplicates with a deviation from the average of below ±10%.

30S Initiation Complex Formation

30S subunits were incubated with MV-mRNA (±SD) and fMet-tRNA, in the absence or presence of all three initiation factors (IF-1, IF-2, and IF-3 in ratio of 1:1:1 or 10:10:10 in relation to ribosomes) and GTP (1 mM).

P_i Complex Formation

70S ribosomes were incubated in the presence or absence of MF-mRNA with charged tRNA (fMet-RNA or AcPhe-RNA) at 37°C for 15 min.

A-Site Binding and Formation of Pretranslocation Complexes

70S ribosomes were first incubated at 37°C for 15 min with mRNA [MF/MV, or poly(A)] and 0.6 μM fMet-tRNA or uncharged tRNA (deacylated $tRNA_f^{Met}$ or $tRNA^{Lys}$), in order to prefill the P site. Next, enzymatic A-site binding was performed by addition of a preincubated (2 min at 37°C) ternary complex consisting of EF-Tu (1.2 pmol per pmol tRNA), GTP (0.5 mM), and Phe-tRNA (1.7 × ribosomes), or nonenzymatic binding by addition of the appropriate charged tRNA (AcPhe-tRNA, AcVal-tRNA, or AcLys-tRNA at a final concentration of 0.5 μM). All reactions were then incubated at 37°C for an additional 30 min.

Posttranslocation Complexes

Following formation of pretranslocation complexes (as previously described), EF-G (final concentration of 0.3 pmoles/pmol 70S) and GTP (0.12 mM) were added and the incubation continued for 10 min at 37°C.

Isolation of Posttranslocation Complexes and Addition of Ternary Complex

The posttranslocation complexes were formed as described above and isolated free from ligands (EF-G, GTP, tRNAs, etc.), via centrifugation through a 10% sucrose cushion at 65000 × *g* for 18 hr at 4°C.

Polymerization Systems

Poly(U)-Dependent Poly(Phenylalanine) Formation

The assay conditions had the same final ionic conditions ($H_{20}M_{4.5}N_{150}SH_4Spd_2Sp_{0.05}$) as described above for complex formation and were performed in reaction volumes of 15 μl (or multiples thereof). Each incubation mixture contained 70S ribosomes (0.22 μM) preincubated with each antibiotic for 10 min at 37°C, fractionated poly(U) mRNA (25 μg), [^{14}C]phenylalanine (7 nmol at 10 dpm/

pmol), $tRNA^{Phe}$ (40 pmol), ATP (3 mM), GTP (1.5 mM), acetyl-phosphate (5 mM), and S-100 fraction. The reaction mixture was incubated at 37°C for specified periods of time (up to 60 min), and stopped by hot TCA precipitation as described by Bommer et al. (1996). The Phe incorporation was expressed as pmols of Phe incorporated per pmol 70S ribosomes, as a function of time (Phe/(70S X min)).

Misincorporation Assay

Poly(U)-dependent poly(Phe) was performed as above except in addition 100 pmoles [^{3}H]Leucine (3000 dpm/pmol) and 200 pmol tRNA bulk were included. After incubation and hot TCA precipitation, the radioactivity resulting from [^{14}C]phenylalanine and [^{3}H]leucine isotopes could be accurately separated by scintillation counting. Misincorporation is expressed in terms of leucine molecules incorporated per 1000 molecules of phenylalanine polymerized.

Poly(A)-Dependent Poly(Lys) Assay

This assay was performed as described for poly(U)-dependent poly(Phe) assay, except that poly(A) mRNA (25 μg) replaced poly(U), 2500 pmol [^{14}C]Lysine (120 dpm/pmol) were used in place of Phe, and 1 A_{260} unit of tRNA bulk was added, instead of $tRNA^{Phe}$. Furthermore, after incubation, a hot TCA precipitation was performed, using 5% TCA supplemented with 0.25% sodium wolframate.

All single determinations were performed in duplicates with a deviation from the average of below ±10%.

Antibiotics

Edeine and streptomycin were soluble in water, whereas tetracycline and especially pactamycin required ethanol for stock concentrations. Since high antibiotic concentrations were tested in many cases (up to 200 μM) this meant that a small amount of ethanol up to 2% was present in these cases. With regard to the in vitro translation systems, we saw that 2% ethanol significantly stimulated poly(Phe) and poly(Lys) formation by up to 20% and 35%, respectively, and increased the misincorporation level in the former system from 1/2300 to 1/1900.

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