Effect of Mutations in the A Site of 16 S rRNA on Aminoglycoside Antibiotic-Ribosome Interaction

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Decoding of genetic information occurs upon interaction of an mRNA codon-tRNA anticodon complex with the small subunit of the ribosome. The ribosomal decoding region is associated with highly conserved sequences near the 3’ end of 16 S rRNA. The decoding process is perturbed by the aminoglycoside antibiotics, which also interact with this region of rRNA. Mutations of certain nucleotides in rRNA reduce aminoglycoside binding affinity, as previously demonstrated using a model RNA oligonucleotide system. Here, predictions from the oligonucleotide system were tested in the ribosome by mutation of universally conserved nucleotides at 1406 to 1408 and 1494 to 1495 in the decoding region of plasmid-encoded bacterial 16 S rRNA. Phenotypic changes range from the benign effect of U1406 → A or A1408 → G substitutions, to the highly deleterious 1406G and 1495 mutations that assemble into 30 S subunits but are defective in forming functional ribosomes. Changes in the local conformation of the decoding region caused by these mutations were identified by chemical probing of isolated 30 S subunits. Ribosomes containing 16 S rRNA with mutations at positions 1408, 1407 + 1494, or 1495 had reduced affinity for the aminoglycoside paromomycin, whereas no discernible reduction in affinity was observed with 1406 mutant ribosomes. These data are consistent with prior NMR structural determination of aminoglycoside interaction with the decoding region, and further our understanding of how aminoglycoside resistance can be conferred.

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Keywords: decoding region; paromomycin; rRNA structure; antibiotic resistance

Introduction

Decoding is the process in which a correct interaction between the mRNA codon and the tRNA anticodon is recognized by the ribosome. Decoding occurs on the small (30 S) subunit of the prokaryotic ribosome in a region of highly conserved sequences near the 3’ end of 16 S rRNA (decoding region; Figure 1; Cunningham et al., 1992a,b, 1993; Prince et al., 1982; Zimmermann et al., 1990). This region of rRNA is critical for regulating interaction of the ribosome with different sets of ligands during the various steps of translation (Green & Noller, 1997; Noller, 1991). The decoding region likely adopts unique conformations during the various steps of translation, and this conformational flexibility is critical to ribosome function (Lodmell & Dahlberg, 1997). The near universal conservation of all the nucleotides in the decoding region (Gutell, 1994) suggests that the function of this region of the ribosome is dependent on the rRNA sequence. In addition to the ligands of the translation process (tRNA, mRNA, and protein factors), the decoding region also interacts with the aminoglycoside antibiotics.

The aminoglycosides are a group of related antibiotics that bind to the small ribosomal subunit, causing codon misreading and inhibiting translocation (Davies & Davis, 1968; Davies et al., 1965; Gale et al., 1981). These antibiotics bind directly to 16 S rRNA in the A site of the decoding region (Moazed & Noller, 1987). The aminoglycoside-ribosome interaction has been studied using an oligonucleotide model system that mimics both the affinity and specificity of the interaction of aminoglycosides with the 30 S subunit (Recht et al., 1996). Chemical modification-interference (Blanchard et al.,
and footprinting experiments (Recht et al., 1996; Miyaguchi et al., 1996) with oligonucleotide sequence variants have identified the nucleotides critical for the high-affinity binding of paromomycin to the A-site oligonucleotide indicated in outline (Recht et al., 1996); uridine or guanosine at position 1495 allow high-affinity binding of paromomycin to the A-site oligonucleotide. Mutations that create allele-specific priming site V are indicated (Powers & Noller, 1993). The mutations in the decoding region tested in this work are listed. Nucleotides 1404-1407 and 1492-1497 are universally conserved in all non-mitochondrial 16 S-like rRNA (Gutell, 1994).

The structures of paromomycin and gentamicin-oligonucleotide complexes have been determined by NMR spectroscopy (Fourmy et al., 1996; Yoshizawa et al., 1998). In these structures, the aminoglycosides form specific hydrogen bonds with nucleotides that were identified as critical for high-affinity binding (U1495, C1407-G1494). These nucleotides present hydrogen bond acceptors at the correct distance along the major groove to interact with hydrogen bond donors (1-amino, 3-amino) on ring II (2-deoxystreptamine) of the aminoglycosides (Figure 2). In addition, the binding pocket for the antibiotic is formed by the non-canonical A1408-A1493 base-pair and a bulged nucleotide at position 1492, which are required for binding of paromomycin to the oligonucleotide (Recht et al., 1996).

The structures of the A-site RNA-aminoglycoside complexes provide a framework to test drug-RNA contacts within the context of intact ribosomal subunits. Aminoglycoside antibiotics interact with conserved nucleotides in 16 S RNA, and the role of these nucleotides in ribosome function is not known. Here, we have introduced mutations into
the decoding region in plasmid-encoded copies of 16 S rRNA and expressed these in *Escherichia coli*. The phenotypic effect of rRNA mutations was determined, and mutant 30 S subunits were biochemically characterized. Performing in vitro experiments on isolated 30 S subunits, we observed changes in the conformation of the decoding region in the mutant ribosomes. Finally, we demonstrate that the prior structural and biochemical results on the model oligonucleotide successfully predict the effects of these mutations on aminoglycoside interaction with 30 S subunits.

### Results

#### Phenotype of 16 S rRNA mutations

The *E. coli* genome contains seven copies of the ribosomal RNA (*rrn*) operon, making knockout experiments difficult. To address the effect of rRNA mutations, and to allow purification of mutant ribosomal particles, mutant rRNAs can be expressed from a plasmid, which results in a mixture of wild-type and mutant ribosomes. Here, mutations were introduced into the highly conserved 1400-1500 region of 16 S rRNA (Figure 1) by site-directed mutagenesis, and rRNAs were expressed from either plasmid pKK3535 (Brosius *et al.*, 1981) or pLK35 (Douthwaite *et al.*, 1989) in *E. coli* strain DH1. In addition to the mutations in the decoding region, the allele-specific priming site V was introduced (Powers & Noller, 1993). Priming site V allows exclusive monitoring of the plasmid-encoded rRNA in the mixture of plasmid and genomic encoded 16 S rRNA.

The two plasmids systems yield different levels of expression of mutant rRNAs. We attempted to express all mutations from pKK3535, which contains the natural *rrnB* promoters. Mutations that caused a lethal phenotype when expressed from pKK3535 were expressed from pLK35, which contains the *rrnB* operon under control of the lambda P₈ promoter. Mutant 16 S rRNA in 30 S subunits typically accounted for over 60% of the total rRNA when expressed from the *rrnB* promoters in pKK3535, whereas this proportion was less than half the total rRNA when expressed from the deregulated P₈ promoter in pLK35 (Table 1).

The priming site V mutation is silent (Powers & Noller, 1993) and could be expressed from pKK3535. In addition, the 1406A and 1408G mutations produced viable cells when expressed from the *rrnB* promoters in pKK3535. All other mutations of the decoding region could not be cloned into pKK3535, indicating that these mutations were lethal in *E. coli* when they accounted for the majority of 16 S rRNA in the cell. All of these mutations could be expressed from pLK35 in the absence of the repressor, indicating that the cells tolerated the mutant 16 S rRNA when it comprised 20-45% of the total 16 S rRNA.

The incorporation of mutant 16 S rRNAs into actively translating ribosomes was investigated by sucrose gradient fractionation of ribosomal particles from growing cells. The amounts of plasmid-encoded mutant 16 S rRNA was assayed in the 30 S, 70 S, and polysome peaks. The non-lethal mutations (priming site V, 1406A, and 1408G) typically accounted for over 60% of the total 16 S rRNA (Figure 2).

#### Table 1. Per cent of plasmid-encoded 16 S rRNA present in various fractions of polysome profile

<table>
<thead>
<tr>
<th>16 S rRNA sequence</th>
<th>Lysate</th>
<th>30 S</th>
<th>70 S</th>
<th>Disomes</th>
<th>Trisomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type control</td>
<td>65</td>
<td>65</td>
<td>66</td>
<td>59</td>
<td>62</td>
</tr>
<tr>
<td>1406A</td>
<td>62</td>
<td>69</td>
<td>56</td>
<td>51</td>
<td>50</td>
</tr>
<tr>
<td>1406G</td>
<td>22</td>
<td>20</td>
<td>5</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td>1408G</td>
<td>61</td>
<td>51</td>
<td>66</td>
<td>58</td>
<td>58</td>
</tr>
<tr>
<td>1495A</td>
<td>45</td>
<td>56</td>
<td>31</td>
<td>20</td>
<td>17</td>
</tr>
<tr>
<td>1495G</td>
<td>40</td>
<td>71</td>
<td>31</td>
<td>30</td>
<td>16</td>
</tr>
<tr>
<td>1495C</td>
<td>46</td>
<td>58</td>
<td>48</td>
<td>32</td>
<td>22</td>
</tr>
<tr>
<td>1407G/1494C</td>
<td>30</td>
<td>20</td>
<td>23</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Wild-type (pLK35)</td>
<td>31</td>
<td>33</td>
<td>36</td>
<td>36</td>
<td>40</td>
</tr>
</tbody>
</table>

The amount was determined using priming site V as the marker for plasmid-encoded 16 S rRNA. Wild-type (priming site V), 1406A, and 1408G 16 S rRNAs were expressed from the *rrnB* promoters in pKK3535. The other mutations were presumably lethal in this system, and were therefore expressed from the deregulated P₈ promoter in pLK35. The wild-type (priming site V) sequence was also expressed in pLK35. Values reported are the average of at least two experiments. Disomes and trisomes are fractions from the sucrose gradient containing two and three ribosomes per an mRNA, respectively.
were present at essentially equal levels in 30 S subunits, 70 S monosomes, and polysomes (Table 1). U1406 is universally conserved in all small subunit ribosomal RNAs, whereas position 1408 is an adenine in all prokaryotic rRNAs and a guanine in all eukaryotic rRNAs (Gutell, 1994).

In contrast to the viable 1406A mutation, the 1406G mutation was lethal when expressed in E. coli from pKK3535 and was underrepresented in the actively translating pool of ribosomes. The exclusion of the 1406G rRNA from 70 S ribosomes suggests that this mutation has a detrimental effect on either subunit association or some other step of initiation. The level of expression of 1406G from pLK35 was the lowest of all the mutations tested (22 % versus 40-45%).

Ribosomal RNAs containing any mutation of the universally conserved U1495 were underrepresented in 70 S monosomes and polysomes compared with 30 S subunits. In particular, the 1495A and 1495G rRNAs were enriched in the free 30 S pool relative to the 70 S and polysome fractions. In addition, expression of the 1495G mutation caused an increase in the relative amount of free subunits versus 70 S monosomes observed in the gradient (data not shown).

The 1407G + 1494C 16 S rRNA is present at equal levels in 30 S subunits, 70 S monosomes, and polysomes. The fraction of mutant 16 S rRNA present with this mutation (30 %) is lower than the 1495 mutations expressed from pLK35.

Although many of the mutations to highly conserved nucleotides of the A site were detrimental to ribosome function, the binding of paromomycin to these 30 S subunits could be assayed in vitro. Mutant 30 S subunits were expressed and isolated as a mixture of mutant and wild-type subunits, as described in Methods, and chemical probing experiments were performed in the absence and presence of paromomycin. The priming site V changes in the mutant 16 S rRNA allowed exclusive detection of modifications in mutant RNA.

Perturbations of 16 S rRNA structure caused by decoding region mutations

To assess the effects of A-site mutations on the local structure of 16 S rRNA, chemical probing was performed on the mutant ribosomes in the absence of aminoglycoside antibiotic. Upon introduction of either the 1406A or 1406G mutation, the pattern of chemical modification in the absence of paromomycin to these 30 S subunits could be assayed in vitro. Mutant 30 S subunits were expressed and isolated as a mixture of mutant and wild-type subunits, as described in Methods, and chemical probing experiments were performed in the absence and presence of paromomycin. The priming site V changes in the mutant 16 S rRNA allowed exclusive detection of modifications in mutant RNA.

Binding of paromomycin to mutant 30 S subunits

The binding of the aminoglycoside antibiotic paromomycin to 30 S subunits containing mutant 16 S rRNA was assayed by chemical modification and monitored by primer extension using priming site V. The results are summarized in Table 2. Paromomycin at 10μM concentration protects G1494(N7) and A1408(N1) in wild-type 30 S subunits containing only priming site V mutations (Figure 3(a) and Table 2). The sites of protection and concentration of paromomycin required to observe the footprint are the same as for wild-type 30 S subunits without the priming site V mutation (Moazed & Noller, 1987), indicating that the priming site V mutation does not affect the binding of aminoglycoside antibiotics.

The relative affinities of paromomycin binding to 30 S subunits containing mutations at the uni-
versally conserved U1406-U1495 base-pair were determined. Two mutations were introduced at position 1406; the viable 1406A and the non-viable 1406G. When bound to 1406A mutant 30 S subunits, paromomycin causes a footprint at the same concentration and at the same nucleotides as in wild-type 30 S subunits (Figure 3(b) and Table 2). Likewise, the 1406G mutation has little effect on paromomycin binding. In the presence of 10 μM paromomycin, the same set of nucleotides are protected from chemical modification as in the wild-type 30 S subunits (Figure 3(c) and Table 2).

The importance of the nucleotide at position 1495 for paromomycin binding has been determined using the A-site model oligonucleotide. A uridine (wild-type) or guanosine at this position allows high-affinity binding of paromomycin, while the presence of an adenosine or cytidine at this position disrupts this interaction (Recht et al., 1996). To generalize these results to intact subunits, U1495 was changed to each of the three other nucleotides in 30 S subunits. Paromomycin at 10 μM causes a footprint at G1494(N7) and A1408(N1) in 1495G 30 S subunits, demonstrating

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**Figure 3.** Autoradiographs showing DMS probing experiments with 30 S subunits containing allele-specific priming site V. (a) wild-type, (b) 1406A, (c) 1406G and (d) 1408G. In all experiments 30 S subunits were present at 100 nM. Paromomycin was present at the concentration indicated. Modifications were monitored by primer extension from a DNA oligonucleotide primer complementary to priming site V. G, A, U, and C are dideoxy sequencing lanes. K is extension of unmodified 16 S rRNA.
that this mutation does not disrupt paromomycin binding to 30 S subunits (Figure 4(b)). In contrast, the 1495A mutation greatly reduces amino-glycoside binding to 30 S subunits, and a weak footprint is only observed at 1000 \( \text{mM} \) antibiotic (Figure 4(a)). Finally, a weak footprint at G1494(N7) and A1408(N1) at 100 \( \text{mM} \) paromomycin is observed in 1495C 30 S subunits (Figure 4(c)). This indicates a weaker affinity for these subunits. The universally conserved C1407-G1494 base-pair is critical for the high-affinity binding of paromomycin. When 30 S subunits containing the 1407G ‡ 1494C mutation were modified in the presence of increasing concentrations of paromomycin, no footprint at A1408(N1) was observed in the presence of up to 1000 \( \text{mM} \) antibiotic (Figure 4(d)).

All prokaryotic ribosomes contain an adenine at nucleotide 1408, whereas in all eukaryotic cytoplasmic ribosomes this nucleotide is a guanine. Experiments with the A-site oligonucleotide demonstrated that the presence of an A at position 1408 is critical for high-affinity binding of paromomycin. When paromomycin was incubated with 1408G 30 S subunits, a weak footprint was observed at the N7 of G1494 at 100 \( \text{mM} \) antibiotic (Figure 3(d)). Upon addition of paromomycin there is also weak footprint at the base-pairing face of G1494 in the presence of 100 \( \text{mM} \) antibiotic when 1408G 30 S subunits are probed with kethoxal (data not shown).

Discussion

The interaction of the aminoglycoside paromomycin with the ribosome had been previously characterized using an RNA oligonucleotide model system that corresponds with the A site of the 30 S subunit. The effects of RNA sequence changes on the binding of the antibiotic to the oligonucleotide were determined. Using the results from the oligonucleotide as a guide, the interaction of paromomycin with the A site in the context of 30 S subunits has been perturbed by making mutations of highly conserved nucleotides of 16 S rRNA that lie in the antibiotic binding site. Most mutations of highly conserved nucleotides produced 30 S subunits that were excluded from the pool of actively translating ribosomes. In many cases, the mutant 30 S subunits were deficient in their ability to associate with the 50 S subunit to form 70 S ribosomes. This suggests that the mutations cause either a defect in subunit association or may perturb the interaction with initiation factors. The fraction of mutant 16 S rRNA in the total pool was unusually low for both the 1406G and 1407G ‡ 1494C mutations. This deficiency may be due to instability of the mutant 16 S rRNA. It is unlikely to be caused by stable misfolded 16 S rRNA, as there were no aberrant peaks observed in the polysome profiles of cells expressing any of the mutations.

Table 2. Modification of nucleotides in 16 S rRNA that are protected in the presence of paromomycin in wild-type 30 S subunits

<table>
<thead>
<tr>
<th>DMS modified nucleotide</th>
<th>None</th>
<th>1406A</th>
<th>1406G</th>
<th>1407G + 1494C</th>
<th>1408G</th>
<th>1495A</th>
<th>1495G</th>
<th>1495C</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1408 (N1)</td>
<td>+</td>
<td>++</td>
<td>±</td>
<td>++</td>
<td>nd*</td>
<td>++</td>
<td>++</td>
<td>±</td>
</tr>
<tr>
<td>0 µM</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1 µM</td>
<td>±</td>
<td>-</td>
<td>±</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10 µM</td>
<td>±</td>
<td>-</td>
<td>-</td>
<td>±</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>100 µM</td>
<td>±</td>
<td>-</td>
<td>-</td>
<td>±</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G1491 (N7)</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>0 µM</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>nd*</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1 µM</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10 µM</td>
<td>±</td>
<td>±</td>
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<td>+</td>
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<tr>
<td>100 µM</td>
<td>±</td>
<td>-</td>
<td>-</td>
<td>±</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+++, Strong modification; +, moderate modification; ±, weak modification; --, no modification; nd, reactivity at this nucleotide could not be determined because it is absent in the mutated 16 S rRNA.

* The N7 of G1408 is weakly reactive with DMS and the N1-N2 positions are unreactive with kethoxal.

b The reactivity at the N3 of C1494 could not be determined due to the presence of a reverse transcriptase stop at this nucleotide in the absence of modifying agent. No additional changes were observed at 1000 µM paromomycin.
Pyrimidine-pyrimidine mismatches at this pair cause varying degrees of slow growth rate, whereas purine-purine mismatches are lethal when expressed in *E. coli* (DeStasio & Dahlberg, 1990). These mutations also cause changes in the local conformation of the decoding region (DeStasio *et al.*, 1989).

Mutations of the universally conserved nucleotide U1406 have no dramatic effect on the conformation of this region of rRNA based on the chemical modification pattern of the mutant 30 S subunits (DeStasio *et al.*, 1989). In contrast, mutations at position 1495 affect the modification pattern. The 1495A and 1495G mutations cause a decrease in reactivity at G1494(N7), which is normally very reactive to DMS. This perturbation of the local conformation by mutations at 1495 may be due to the stacking effects of purines 3' to G1494 that propagate down the 1490 stem. Thermodynamic studies of oligonucleotides have demonstrated that 3'-terminal purines have a stabilizing effect on helices, whereas 5'-terminal purines do not (Freier *et al.*, 1985, 1986).

Alterations of the chemical modification of 16 S rRNA in the absence of aminoglycoside indicate that mutations in the A site cause at least a local change in the conformation of the rRNA. The allele-specific priming site V only allows exclusive monitoring of mutant 16 S rRNA in the 1400-1500 region (Powers & Noller, 1993). There may be changes in the conformation of other regions of

![Figure 4. Autoradiographs showing DMS probing reactions with 30 S subunits containing allele-specific priming site V.](image)
16 S rRNA caused by mutations in the decoding region, but these would be difficult to observe in the mixture of mutant and wild-type 30 S subunits. In particular, the 530 loop region has been implicated in decoding function (Powers & Noller, 1994). Binding of neomycin causes an enhancement in reactivity at C525 in the 530 loop (Moazed & Noller, 1987). The effect of the decoding region mutations on modification of the 530 loop could not be determined, since no allele-specific priming site has been successfully engineered for this region (for a discussion, see Powers & Noller, 1993).

In the A-site RNA-aminoglycoside structures, the antibiotic binds in the RNA major groove. The binding pocket for aminoglycosides is formed in part by universally conserved nucleotides, several of which were mutated in this study. Ring I sits in a binding pocket created by a non-canonical A1408-A1493 base-pair that is critical for the high-affinity binding of the antibiotic. Ring II (2-deoxystreptamine) makes sequence specific contacts with the universally conserved N7 of G1494 and the O4 of U1495. All aminoglycosides that bind to the decoding region A site contain rings I and II, which make contacts with the highly conserved nucleotides of 16 S rRNA (Fourmy et al., 1996, 1998). Alteration to the aminoglycoside binding pocket, caused by mutation of the C1409-G1491 base-pair, disrupts the interaction of paromomycin with the ribosome (DeStasio et al., 1989).

A hydrogen bond between U1495(O4) and the 1-amino group of 2-deoxystreptomycin is critical for the high-affinity binding of paromomycin to 30 S subunits. This interaction is mediated by the conserved C1409-G1491 base-pair, which is disrupted by the 1408G mutation (DeStasio et al., 1989). Mutation to a lesser degree 1495C, disrupt the high-affinity binding of paromomycin to 30 S subunits. These results are consistent with the proposed model for the A-site oligonucleotide (Recht et al., 1996).

U1406 does not contact paromomycin directly, and mutations at position 1406 were found to have no effect on the binding of paromomycin to the 30 S subunit. The 1406A and 1406G mutant 30 S subunits bind paromomycin with approximately the same affinity as wild-type 30 S subunits. Ring III of gentamicin and related aminoglycosides contacts U1406 (Yoshizawa et al., 1998) and mutation to A may disrupt high-affinity binding for this class of aminoglycosides. In contrast, mutation to G1494(N7) disrupts the critical interaction in 30 S subunits.

The binding pocket for ring I, formed by the A1408-A1493 base pair, is located in the decoding region A site. U1406 does not contact paromomycin directly, and mutations at position 1406 were found to have no effect on the binding of paromomycin to the 30 S subunit. The 1406A and 1406G mutant 30 S subunits bind paromomycin with approximately the same affinity as wild-type 30 S subunits. Ring III of gentamicin and related aminoglycosides contacts U1406 (Yoshizawa et al., 1998) and mutation to A may disrupt high-affinity binding for this class of aminoglycosides. In contrast, mutation to G1494(N7) disrupts the critical interaction in 30 S subunits.

The precise conformation of the G1408-A1493 base-pair cannot be determined from the chemical modification data, as both the N7 and the N1-N2 hydrogen bond acceptor oxygen, 1495A and, to a lesser degree 1495C, disrupt the high-affinity binding of paromomycin to 30 S subunits. These results are consistent with those observed in experiments with the A-site oligonucleotide (Recht et al., 1996).

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The precise conformation of the G1408-A1493 base-pair cannot be determined from the chemical modification data, as both the N7 and the N1-N2
positions of G1408 are unreactive to the chemical probes. The 1408G mutation causes an increased reactivity of G1494 with kethoxal, suggesting that formation of the C1407-G1494 base-pair is altered by this mutation. Structure determination of the oligonucleotide version of the mutant could reveal the details of this perturbation.

The results presented here demonstrate a precise correlation between nucleotides critical for high-affinity binding of the aminoglycoside paromomycin to 30 S ribosomal subunits and those previously identified as critical for binding to an oligonucleotide model of the decoding region. These results confirm the utility of the model oligonucleotide system for characterizing aminoglycoside-ribosome interaction using biophysical methods.

Mutations have been made in universally conserved nucleotides of the decoding region. None of the mutations are phenotypically silent, and most are lethal when they account for a majority of the total rRNA in the cell. If the high-affinity binding of aminoglycosides to the A site is a prerequisite for antibiotic action, mutations that disrupt this interaction should confer resistance to the antibiotic. This resistance would be dependent upon function of the mutant 30 S subunits, since non-functional aminoglycoside-resistant ribosomes would be lethal. Determining the effects of these mutations on the function of the ribosome will provide insight to the conservation of this sequence from bacteria to mammals.

Methods

Site-directed mutagenesis

Mutations were introduced into the 16 S rRNA gene by site-directed mutagenesis, using the method described by Kunkel et al. (1987), with the modifications for use with BlueScript by Evnin & Craik (1988). Mutations were constructed in pBS-V (T. Powers and H. F. Noller, unpublished results), which contains the Apal-XbaI fragment from pSTL102 (Triman et al., 1989) with allele-specific priming site V. Oligos used for mutagenesis are as follows:

1406A, 5'-ACC-GCC-GCA-CAC-ACC-ATG; 1406G, 5'-ACC-GCC-GCG-CAC-ACC-ATG; 1407G, 5'-CCG-CCC-GTG-ACA-CCA-TG; 1408G, 5'-CCG-CCC-TGG-CAC-CAT-GG; 1494C, 5'-GGG-GTG-AAC-CCA-TGC-GAA-G; 1495A, 5'-GGT-GAA-GAC-GTA-ACA-AG; 1495G, 5'-GGT-GAA-GGC-GTA-ACA-AG; 1495C, 5'-GGT-GAA-GGC-GTA-ACA-AG. The fragment from pSTL102 contains the spectinomycin-resistance mutation (C1192U). All decoding site mutants were made both with and without this additional mutation. All probing experiments were performed on 30 S subunits containing the wild-type (1192C) sequence. Strain XL-1 Blue was used as the recipient of the mutagenesis reactions. Mutations were identified by dideoxy sequencing of the pBS-V derivatives. These mutations were introduced into either pK3535 (1406A, 1408G) or pKL35 (all others) for expression of 16 S RNA using the unique Apal and XbaI sites present in all three plasmids. Strain DH1 was used as the recipient of the final constructs. The presence of the mutations in the final constructs was verified by dideoxy sequencing analysis.

Preparation of 30 S subunits

Mutant 30 S subunits were prepared following a modified procedure of Powers & Noller (1991). One liter cultures in LB media containing 100 μg/ml ampicillin were grown to an A600 of 0.4. The cells were plated on ice for 20 minutes and then pelleted. Cells were washed and resuspended in 15 ml of 20 mM Tris-HCl (pH 7.5), 100 mM ammonium chloride, 10 mM magnesium chloride, 0.5 mM EDTA, 6 mM 2-mercaptoethanol, and lysed by passage through a french press at 1200 psi. The lysate was separated by centrifugation at 30,000 g for 15 minutes. The supernatant (approximately 25 ml) was applied to a 30 ml 1.1 M sucrose cushion and ribosomes were pelleted by centrifugation at 33,000 rpm in a Ti45 rotor for 22 hours.

Subunits were separated by dialyzing 15 mg ribosomes against one liter of subunit dissociation buffer (50 mM Tris-HCl (pH 7.8), 1 mM magnesium chloride, 100 mM ammonium chloride, 6 mM 2-mercaptoethanol) for six hours. The dialyzed subunits were applied to a 10 %-40 % sucrose gradient prepared with a buffer containing 50 mM Tris-HCl (pH 7.8), 10 mM magnesium chloride, 100 mM ammonium chloride, 6 mM 2-mercaptoethanol. The gradients were spun in an SW28 rotor for 16 hours at 25,000 rpm. Fractions were analyzed by using a Brandel gradient fractionator monitoring UV absorbance at 254 nm with an ISCO detector. Fractions containing 30 S subunits were collected. The subunits were pelleted by centrifugation at 45,000 rpm in a Ti70 rotor for 12 hours. Subunits were suspended in the same buffer as used in the sucrose gradients and frozen in small aliquots in liquid N2 and stored at −80 °C.

Preparation of polysomes and quantification of mutant 16 S rRNA

Freshly inoculated 200 ml cultures were grown at 37 °C until an A600 of ≈0.4. Cells were pelleted and polysomes prepared by the method of Ron et al (1966) with the modifications by Powers & Noller (1990). Lysates (approximately ten A260 units) were layered onto 10 ml 10 %-40 % linear sucrose gradients made in a buffer containing 20 mM Tris-HCl (pH 7.8), 10 mM MgCl2, 100 mM NH4Cl, 2 mM dithiothreitol and centrifuged in an SW-41 rotor at 4 °C for 2.5 hours at 35,000 rpm. Gradients were analyzed as above, and 0.15 ml fractions were collected. Ribosomal RNA was isolated by extraction of appropriate fractions (or crude lysate) with phenol/chloroform/isoamyl alcohol (25:24:1). RNA was concentrated by precipitation with ethanol and resuspended in water.

The fraction of plasmid-encoded rRNA was determined by a variation of the method of Sigmund et al. (1988). An 18 nucleotide primer complementary to nucleotides 1541-1523 of 16 S rRNA was 5' end-labeled with [γ-32P]ATP. The primer-rRNA duplex ends two nucleotides downstream from position 1521 of 16 S rRNA, where the wild-type C is replaced by G in plasmid-encoded priming site V RNA. By extending the primer annealed to the rRNA template in the presence of two deoxynucleotide triphosphates (75 μM dGTP, dATP) and two deoxyribonucleotide triphosphates (15 μM ddCTP, ddTTP), the relative proportions of the wild-type and priming site V rRNA were determined from
the intensity of the two different terminated bands. Plasmid-encoded template produces an extension product of +2 nucleotides and genomic-encoded template produces a +3 product. The dyeoxy TTP was included to avoid additional termination products caused by modified nucleotides at positions G1516, A1518, and A1519 which cause the wild-type template to terminate at C1520. The fraction of plasmid-encoded 16 S rRNA was quantitated by exposing the gel on a Molecular Dynamics PhosphorImage screen and analyzed using Molecular Dynamics ImageQuant software.

**Chemical modification**

Modification reactions were performed essentially as described by Moazed & Noller (1987). Wild-type or mutant 30 S subunits (10 pmol) were activated in 100 µl reaction buffer (80 mM potassium cacodylate (pH 7.2) 100 mM ammonium chloride, 20 mM magnesium chloride, 1 mM DTT, 0.5 mM EDTA) by incubation at 42°C for 20 minutes. Following addition of paromomycin (Sigma) and incubation for 30 minutes at 37°C and ten minutes on ice, DMS (2 µl, 1:6 in ethanol) was added. Following incubation at 37°C for ten minutes, 25 µl of 1 M Tris acetate (pH 7.5), 1.5 M sodium acetate, 1 M 2-mercaptoethanol was added. Subunits were concentrated by ethanol precipitation and modified RNA was obtained by extraction three times with phenol and twice with chloroform.

Reduction with sodium borohydride and aniline-induced strand scission were performed as described (Fourmy et al., 1998).

Modification with kethoxal was performed in the same buffer as DMS probing reactions. Following activation of subunits and addition of paromomycin as described above, kethoxal (5 mM, 37 mg/ml in 20% (v/v) ethanol) was added. Subunits were modified for ten minutes at 37°C and potassium borate (pH 7.0) was added to a final concentration of 25 mM. Following precipitation with ethanol, modified RNA was obtained as described above. Modified RNA was suspended in 25 mM potassium borate, pH 7.0.

**Primer extension**

Primer extension from allele-specific priming site V was performed following a modified version of that described by Powers & Noller (1993) and Stern et al. (1988). A 21 nucleotide DNA primer complementary to nucleotides 1535-1514 of priming site V 16 S rRNA was annealed to the rRNA in hybridization buffer (225 mM K-Hepes, 450 mM KCl, pH 7.0) by heating at 90°C for one minute followed by cooling to 50°C. Following annealing of the primer, extension was performed by addition of 2 µl of a mix consisting of 870 mM Tris-HCl (pH 8.5), 67 mM magnesium chloride, 67 mM DTT, 2.0 µCi/µl [α-32P]dTTP, and 0.2 µl AMV reverse transcriptase (approximately 2 units, Seikagaku). Following incubation at 50°C for ten minutes, 1 µl of a solution containing 1 mM each of the four dNTPs was added and the reaction was allowed to proceed for an additional 30 minutes at 50°C. The reaction was stopped by precipitation with ethanol. Following suspension of the cDNA in 10 µl 7 M urea loading dye, 2.5 µl of each sample was run on an 8% polyacrylamide gel.

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**References**


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