Pactamycin Resistance Mutations in Functional Sites of 16 S rRNA

Alexander S. Mankin

Mutants of an archaeon *Halobacterium halobium*, resistant to the universal inhibitor of translation, pactamycin, were isolated. Pactamycin resistance correlated with the presence of mutations in the 16 S rRNA gene of *H. halobium* single rRNA operon. Three types of mutations were found in pactamycin resistant cells, A694G, C795U and C796U (*Escherichia coli* 16 S rRNA numeration) located distantly in rRNA primary structure but probably neighboring each other in the three-dimensional structure. Pactamycin resistance mutations either overlapped (C795U) or were located in the immediate vicinity of nucleotides protected by the drug in *E. coli* and *H. halobium* 16 S rRNA indicating that corresponding rRNA sites might be directly involved in pactamycin binding. Ribosomal functions were not affected significantly either by mutation of C795 (one of the positions protected by the P-site-bound tRNA), or by mutations of A694 and C796 (which neighbor nucleotides protected by tRNA) suggesting that tRNA-dependent protections of C795 and G693 are explained by a conformational change in the ribosome induced by the P-site-bound tRNA. A novel mode of pactamycin action is proposed suggesting that pactamycin restricts structural transitions in 16 S rRNA preventing the ribosome from adopting a functional conformation induced by tRNA binding.

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Antibiotics are important tools for studying mechanisms of protein synthesis (Vazquez, 1979; Cundliffe, 1981). Bound in the vicinity of the ribosomal functional centers, antibiotics either affect ribosome conformation or interfere with binding or dissociation of ribosomal substrates. Though most inhibitors of translation are specific to ribosomes of a particular type, there is a relatively limited subset of antibiotics which can inhibit protein synthesis in all three evolutionary domains: Eucarya, Bacteria and Archaea (Woese et al., 1990). Such drugs are presumed to interact with highly conserved ribosomal centers that remain unchanged throughout the course of evolution. Knowledge of where and how such universal antibiotics interact with the ribosome is important for understanding the fundamental principles of ribosome organization and function.

Pactamycin (Pct) inhibits protein synthesis in all studied organisms (Goldberg, 1975; Mankin & Garrett, 1991). The drug interacts with the small ribosomal subunit and blocks translation primarily by interfering with initiation of protein synthesis (Goldberg, 1975; Cundliffe, 1981). Interaction with rRNA is essential for Pct binding to the ribosome. Pct protects G693 and C795 in *Escherichia coli* 16 S rRNA from chemical modification (Egebjerg & Garrett, 1991; Woodcock et al., 1991). Furthermore, in Pct producer *Streptomyces pactum*, methylation of A964 (*E. coli* numeration) in 16 S rRNA renders ribosomes resistant to the drug (Ballesta & Cundliffe, 1991). It remained unclear, however, whether any of these sites in rRNA is directly involved in drug binding.

Drug resistance mutations define the site of antibiotic action. However, isolation of mutations in rRNA is complicated by the presence of multiple copies of rRNA genes in most organisms. One way to overcome this problem is to use an organism with a single set of rRNA genes. An
archaeon *Halobacterium halobium*, which possesses only one copy of the chromosomal rRNA operon (Hofman *et al.*., 1979; Mankin & Kagramanova, 1986), has been used previously to isolate rRNA mutations conferring resistance to antibiotics targeted against the large ribosomal subunit (reviewed in (Mankin, 1995)). Isolation of *H. halobium* Pct' mutants and mapping the resistance mutations are described in this paper. Studies of the mutants provided information about the functional significance of rRNA sites involved in interaction with the drug and insights into the mode of Pct binding and action. For convenience of comparison, *E. coli* numeration of rRNA positions is used in this paper.

**Isolation of Pct' mutant of *H. halobium***

Growth of halophilic archaeon *H. halobium* is significantly slowed at 1 μM Pct and practically abolished at 10 μM. Pct' mutants of *H. halobium* were selected on agar plates containing 40 μM Pct. Pct' mutants appeared with a frequency of ca. 10⁻⁷, forming colonies of various size. Fourteen individual colonies from the plate were grown in liquid cultures, and mutations were mapped by direct sequencing of the 16S rRNA. Three types of mutations were identified. The C795U mutation was found in large colonies, the A694G mutation was present predominantly in medium size colonies, and the C796U mutation was identified in two smallest colonies (Figure 1). The presence of mutations only slightly reduced cell growth rate in the absence of antibiotic. While doubling time of the wild-type culture was 7.2 hours, it increased to 7.8 hours for the C795U mutant, to 8.1 hours for the A694G mutant and to 8.3 hours for the C796U mutant. At the same time, the level of antibiotic resistance conferred by different mutations was approximately the same; all three types of mutants, C795U, A694G and C796U, could grow at ten times higher concentration of the drug than wild-type cells (Figure 2). Therefore, sizes of mutant colonies most probably reflected differential effects of mutations on cell growth rate rather than antibiotic sensitivity.

![Figure 1. Pct' mutations in 16S rRNA of *H. halobium*. The central and 3' major domains of *H. halobium* 16S rRNA are shown. Nucleotide numeration (shown only for hairpins 23, 25 and 33 (Van de Peer *et al.*, 1997)) is that of *E. coli* 16S rRNA. Pct' mutations are shown by closed arrows. Nucleotides protected by Pct from chemical modification are marked by round dots and positions protected by the P-site-bound tRNA are circled (tRNA-related protections are shown only for the hairpins 23 and 25). A964 in hairpin 33, whose methylation renders ribosomes of Pct-producer resistant to the antibiotic is indicated a by black rectangle. For isolation of the mutants, 2 x 10⁸ cells of *H. halobium*, strain R1 (Stoeckenius & Kunau, 1968), were plated onto agar medium containing 40 μM Pct and plates were incubated 10 days at 37°C. Individual colonies that appeared on the plate were inoculated into 3 ml liquid medium containing 5 μM Pct and grown to A₅₅₀ = 0.8. Cells were pelleted, total RNA was isolated and 16S rRNA was sequenced by reverse transcriptase-mediated primer extension as described previously (Stern *et al.*, 1987) with modifications described in (Mankin & Garrett, 1991).](image-url)
Introducing Pct\(^\prime\) mutation into the chromosomal 16 S rRNA gene of wild-type H. halobium

In order to verify whether a single nucleotide alteration in the 16 S rRNA is sufficient to render cells Pct resistant, the C795U mutation was introduced into the 16 S rRNA gene of the single chromosomal rRNA operon of wild-type H. halobium (Hofman et al., 1979). The C795U mutation was engineered in the 16 S rRNA gene in the pHRZH plasmid (Mankin et al., 1992), which contains the entire H. halobium rRNA operon with two thiostrepton-resistance (Thsr) mutations (A1067G and A1095G) and an anisomycin-resistance (Anir) mutation (C2452U) present in the 23 S rRNA gene. The resulting plasmid, pHRZHP2, was transformed into wild-type H. halobium using the described procedure (Cline et al., 1989) and transformants which acquired resistance to thiostrepton and anisomycin were selected. pHZH (and pHZHP2) is unstable in the cell either as a free plasmid or in the chromosome-integrated form (Mankin, 1995) and is rapidly lost. Hence, transformed cells can become Thsr\(^\prime\)/Anir\(^\prime\) only by exchanging homologous fragments between the wild-type chromosomal rRNA operon and plasmid-borne mutant rRNA genes prior to loss of the plasmid. If the rRNA segment, exchanged between the plasmid and the chromosome, includes the 3\(^{\prime}\) half of the 16 S rRNA gene, then transformed cells acquire not only selective 23 S rRNA mutations but also the C795U mutation in the 16 S rRNA. A clone with the C795U mutation was found by sequencing the segment of 16 S rRNA close to position 795 in several transformants selected on an anisomycin/thiostrepton plate. When grown in liquid culture in the presence of Pct, this clone exhibited Pct resistance similar to that of the originally selected C795U mutant (Figure 3). In contrast, the transformants that acquired anisomycin and thiostrepton-resistance mutations in the 23 S rRNA, but did not have the C795U mutation in the 16 S rRNA, remained sensitive to the drug. Since transformed cells were not exposed to Pct during transformation and selection, appearance of any second site mutation contributing to Pct resistance was highly unlikely. It can be concluded, therefore, that the presence of C795U mutation is sufficient to render cells resistant to Pct.
Of the three identified Pct mutations, only C795U was introduced into the wild-type chromosome using the pHRZH-based transformation system. However, the conclusion that a single mutation is sufficient for Pct resistance can be extrapolated to the other two Pct mutations because the frequency of appearance of Pct mutants carrying A694G or C796U mutations is comparable with that of the C795U mutant and corresponds to the frequency of single point mutations in H. halobium (Hummel & Böck, 1987; Mankin & Garrett, 1991). If a second site mutation was required to render A694G or C796U mutants Pct-resistant, such mutants would appear with a much lower frequency.

Pct mutations and ribosome function

Pct mutations described in this paper are located in the segments of rRNA implicated as the sites of ribosome–tRNA interactions (Moazed & Noller, 1986, 1990; Döring et al., 1994). The P-site bound tRNA protects a specific set of nucleotides in the 16S rRNA from chemical modification, among them G693 and C795. If these positions are essential for tRNA binding, then C796U and A694G mutations, located in the immediate vicinity of the protected nucleotides, and especially the C795U mutation, which directly affects a nucleotide protected by tRNA, are expected to impair tRNA binding and to be highly deleterious to the cell. However, filter binding did not show difference in affinity of tRNA to the P-site of the wild-type and mutant H. halobium ribosomes (data not shown), while the generation time of the mutant cells was only slightly increased compared to the wild-type. It can be concluded that Pct mutations, including C795U, introduced no significant defect in ribosomal functions suggesting that the identity of rRNA bases at these positions is not critical for tRNA binding. Similar conclusions were drawn from modification interference experiments (von Ahsen & Noller, 1995) where 30S subunits modified at C795 or G693 could still bind tRNA in the P-site demonstrating that the integrity of C795 and G693 bases were not essential for the tRNA binding. Consequently, protection of these nucleotides by tRNA is better explained by a conformational change induced in the 16S rRNA upon tRNA binding rather than by direct contact of these nucleotides with tRNA.

Hairpins 23 and 25 are thought to be implicated in subunit association. It was shown previously that E. coli ribosomes carrying mutations at positions C791 and A792 in the hairpin 25 were defective in subunit association and binding of the initiation factor IF3 resulting in decreased growth rates of the mutant cells containing mixed populations of the wild-type and mutant ribosomes (Tapprich et al., 1989; Santer et al., 1990). Since neither of the Pct mutations significantly affect growth rate of the mutant H. halobium cells with a homogeneous population of mutant ribosomes, it can be concluded that the mutated positions (both in hairpins 23 and 25) are not essential for subunit association or binding of initiation factors.

Viability of cells carrying the C795U mutation raises a question of the importance of conserved nucleotides in rRNA. C795 is invariant in all the sequenced bacterial and archaeal 16S rRNAs and eukaryotic 16S-like rRNAs (Maidak et al., 1997). Such high conservation is usually perceived as an indicator of functional importance of the nucleotide. Nevertheless, mutation of C795, similar to mutations of some other conserved nucleotides (Levie et al., 1994; Triman, 1997), produced surprisingly small effects on cell growth, and thus, on protein synthesis. Therefore, C795 is not absolutely crucial for ribosomal functions. Instead, this nucleotide may play an accessory role facilitating one of the ribosomal reactions. Similarly, other conserved rRNA nucleotides whose mutations do not significantly impair cell growth may also only assist in some ribosome activities without being crucially important.

Footprinting Pct on the wild-type and mutant ribosomes

Pct protects G693 and C795 in E. coli 16S rRNA from chemical modification (Egebjerg & Garrett, 1991; Woodcock et al., 1991). RNA footprinting was used to study effects of Pct mutations on rRNA structure and the interaction of Pct with the ribosome. H. halobium ribosomes isolated from the wild-type or mutant cells were modified by kethoxal or dimethyl sulfate in the absence or in the presence of Pct, and base modification patterns were analyzed by primer extension (Stern et al., 1987). In the absence of the drug, the A694G mutation did not produce any notable change in modification patterns either in the vicinity of the mutation or in distant rRNA regions (Figure 4). Neither C795U, nor C796U mutations affected base modifications in the loop of hairpin 23. In contrast, the C795U mutation increased accessibility of nucleotides in the hairpin 25 loop to chemical modification. A787, A790, G791, A792 and A794 were modified to a higher extent in the C795U mutant compared to the wild-type ribosome or to the A694G mutant (Figure 4A and B). Similarly, the C796U mutation also increased accessibility of bases in the loop of hairpin 25 (not shown). Primer extensions on the 23S rRNA, prepared from the C795U mutant, reproducibly showed the presence of two bands corresponding to G799 and G800 (see Figure 4A and B). Since the same bands were present in the control unmodified samples, they did not reflect an altered modification pattern, but were likely caused by reverse transcriptase pausing “in front” of the apex part of hairpin 25, which in the C795U mutant is more stable due to an additional A-U base pair introduced by the mutation.

Binding of Pct to the E. coli ribosome shields N1 and N7 positions of G693 and N3 of C795 in 16S
Figure 4. Accessibility of nucleotides in *H. halobium* wild-type and mutant ribosomes to kethoxal (A) and dimethyl sulfate (B) modification in the absence and in the presence of Pct. Lanes A, G and C, products of dideoxy sequencing of 16S rRNA; lane K, unmodified RNA; lane 0, ribosomes modified in the absence of Pct; lanes 10 and 100, ribosomes modified in the presence of 10 and 100 μM Pct, respectively. Positions of nucleotides whose modification is affected by Pct are shown by arrows. Nucleotides which become more accessible to chemical modification in the C795U mutant are shown by arrowheads. A strong reverse transcriptase stop seen in the C795U mutant at positions 799 and 800 in all lanes, including unmodified RNA (lane K) is not reflecting any change in accessibility of nucleotides to chemical modification. Ribosomes were isolated from the wild-type or Pct‘ mutant *H. halobium* cell as described previously (Tan et al., 1996). An amount (10 pmol) of ribosomes was incubated for ten minutes at 37°C with 0.5 nmol or 5 nmol Pct in 50 μl reaction buffer (80 mM potassium cacodylate, pH 7.2/3 M KCl/100 mM MgCl₂/1.5 mM DTT). Kethoxal (2 μl, 50% in ethanol) or dimethyl sulfate (10% in ethanol) was added and incubation was continued for ten minutes at 37°C. This was followed by addition of 300 μl cold stop buffer (0.3 M sodium acetate, 75 mM potassium borate, pH 6.5, for kethoxal modification, or 0.3 M sodium acetate, 25 mM mercaptoethanol, pH 5.5, for dimethyl sulfate modification). Tubes were briefly vortexed and 1 ml cold ethanol was added. Ribosomes were precipitated, RNA was extracted and analyzed by primer extension as described (Stern et al., 1987).
rRNA from modification with kethoxal and dimethyl sulfate (Egebjerg & Garrett, 1991; Woodcock et al., 1991). Similarly, a strong Pct-dependent footprint was observed in the kethoxal-modified *H. halobium* ribosome at G693 (N1 position) (Figure 4A). Pct also protected C795 and G693 in *H. halobium* 16 S rRNA from modification with dimethyl sulfate (Figure 4B). Though no attempt was made to reveal protections of guanines at N7 by aniline cleavage in the dimethyl sulfate-modified samples, a band corresponding to G693 was present, probably reflecting reverse transcriptase pausing at the N7-methylated G693; this pausing became less pronounced when ribosomes were modified in the presence of Pct (see Figure 4B). Thus, Pct protects the same set of nucleotides in the wild-type *H. halobium* ribosomes as in *E. coli*. However, when mutant *H. halobium* ribosomes, carrying either A694G, C795U or C796U mutations, were incubated with the drug, no distinct Pct-dependent protections were observed (Figure 4) (C796U mutant data not shown). The C795U mutation, which itself involved the nucleotide residue protected by Pct, completely abolished Pct footprint at G693, while A694G or C796U mutations eliminated Pct footprints at both G693 and at C795. The most straightforward explanation of these results is that A694G, C795U and C796U mutations prevent or, at least, significantly reduce binding of Pct to the ribosome resulting in the lack of drug-dependent protections.

**Site of interaction of Pct with 16 S rRNA**

The broad spectrum of organisms affected by Pct suggests that it interacts with a universally conserved ribosomal structure. Three found Pct mutations were located in two distinct sites in the 16 S rRNA. Two general models can account for occurrence of drug resistance mutations widely spaced in the rRNA primary structure. One possibility is that the drug directly interacts with both rRNA sites and mutations in either site prevent or significantly reduce drug binding by eliminating one of the contact points. Alternatively, mutations in one or both sites can induce an allosteric change in rRNA conformation preventing interaction with the drug; in this case, binding does not necessarily occur in the immediate vicinity of the mutated nucleotide. The experimental data agree better with the first model. Though both C795U and C796U mutations induce a local conformational change increasing accessibility of bases in the adjacent loop to chemical modifications, neither of these mutations, nor A694G, produces any notable long-distance effects in the 16 S rRNA modification pattern in absence of the drug. Hence, it is likely that Pct mutations affect nucleotides that are either directly involved in interaction with the drug or located in the immediate vicinity of the drug binding sites. In the loop of the helix 23, the A694G mutation neighbors G693 which is protected by Pct from chemical modification (Egebjerg & Garrett, 1991; Woodcock et al., 1991). Accordingly, one or both of these nucleotides may be involved in interaction with Pct. In the loop of the helix 25, Pct protects C795 from modification with dimethyl sulfate, while mutations of C795 and C796 abolish drug binding. Therefore, C795 and C796 are the primary candidates for forming direct contacts with the drug molecule. At the same time, since C795U and C796U mutations also “open” the entire loop increasing accessibility of several nucleotides to chemical modification (shown by arrowheads in Figure 4), it is possible that other nucleotides in the loop may interact with Pct. Simultaneous interaction of Pct with nucleotides in the loops of helices 23 and 25 agrees with their juxtapositioning in the current models of the 16 S rRNA three-dimensional structure (Stern et al., 1988; Brimacombe et al., 1988). These rRNA segments can be crosslinked in the small ribosomal subunit (Atmadja et al., 1986) and sugar-phosphate backbones in both loops are protected from Fe²⁺/EDTA cleavage by ribosomal protein S11 (Powers & Noller, 1995).

The third 16 S rRNA site that is related to Pct action is A964 whose methylation renders ribosomes from *S. pactum*, a producer of Pct, resistant to the drug (Ballesta & Cundliffe, 1991). However, in footprinting experiments, A964 was inaccessible for modification with dimethyl sulfate both in *E. coli* and *H. halobium* ribosomes, and Pct mutations in hairpins 23 and 25 did not change the nucleotide modification pattern in the vicinity of A964 (not shown). Therefore, it was not possible to conclude whether A964 interacts directly with Pct or its methylation in *S. pactum* renders ribosomes Pct-resistant via an allosteric effect.

Though Pct can be cross-linked to ribosomal proteins in evolutionary distant ribosomes (Tejedor et al., 1985, 1987), previous and newly obtained data clearly implicated 16 S rRNA in drug binding. It was tempting to test whether Pct can interact with naked 16 S rRNA since binding of some antibiotics to rRNA was demonstrated previously (Thompson et al., 1979; Purohit & Stern, 1994). Unfortunately, all attempts to detect Pct footprints on naked *E. coli* or *H. halobium* 16 S rRNA were unsuccessful (data not shown). It may well be that proper conditions which would allow 16 S rRNA to adopt a conformation competent for drug binding were not found. Alternatively, ribosomal proteins may facilitate drug binding either directly or by stabilizing rRNA functional conformation.

**Mode of action of Pct**

Pct inhibits initiation of translation. Both Pct and P-site bound tRNA protect G693 and C795 in 16 S rRNA from chemical modification. Since initiation of translation includes binding of tRNA to the P-site of the small ribosomal subunit, it was proposed that overlap of footprints produced by tRNA and Pct “can account in a straightforward
way for the mode of action of the drug (Woodcock et al., 1991). However, as discussed above, while Pct appears to interact directly with rRNA in the vicinity of G693 and C795, tRNA footprints at these positions is better explained by a conformational change of 16S rRNA induced by binding of tRNA in the ribosomal P-site. Accordingly, Pct may interfere with P-site related translation initiation events in a less straightforward way than it was thought before. An interesting possibility is that Pct prevents 16S rRNA from undergoing a structural transition associated with tRNA binding in the P-site. As follows from comparison of tRNA footprinting (Moazed & Noller, 1986, 1990) and modification interference data (von Ahesen & Noller, 1995), such a transition may involve the relative movement of helices 23 and 25. The Pct molecule, binding simultaneously to RNA in the loops of hairpins 23 and 25, may lock them into one particular conformation thereby preventing their movement. This model can explain an observation that tRNA-dependent binding of initiator tRNA to eukaryotic ribosomes is not affected by Pct, but the conversion of the complex into a puromycin reactive form is inhibited (Goldberg, 1975; Seal & Marcus, 1972). A similar mode of action, based on prevention of dynamic structural transitions in rRNA, may also apply to other inhibitors of translation (B. T. Porse, I. Leviev, A. S. Mankin & R. A. Garrett unpublished results).

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References


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