Mutations in the Peptidyl Transferase Center of 23 S rRNA Reveal the Site of Action of Sparsomycin, a Universal Inhibitor of Translation

Ghee T. Tan, Alexandra DeBlasio and Alexander S. Mankin*

Center for Pharmaceutical Biotechnology-m/c870
University of Illinois, 900 S. Ashland Avenue, Chicago IL 60607-7173, USA

Sparsomycin is a universal and powerful inhibitor of peptide bond formation which, in contrast to many other ribosome-targeted antibiotics, does not produce footprints on rRNA. A mutant of an archaeon Halobacterium halobium has been isolated that exhibits resistance to sparsomycin. Resistant cells possessed a mutation in the 23 S rRNA, where C2518 (C2499 in Escherichia coli) was substituted by U. Introduction of the C2518U mutation into the chromosomal 23 S rRNA gene of wild-type H. halobium rendered cells resistant to sparsomycin, demonstrating that a single nucleotide alteration in the rRNA is sufficient to confer resistance. Accordingly, ribosomes containing mutant 23 S rRNA exhibited increased tolerance to sparsomycin in vitro. Mutations of two other nucleotide positions in the peptidyl transferase center, C2471 and U2519 (C2452 and U2500 in E. coli), conferred resistance to low concentrations of sparsomycin. The location of the sparsomycin resistance mutations reveals the possible site of drug binding and/or action. Our findings provide further support for the idea that rRNA may be directly involved in interaction with antibiotics and the catalysis of the peptide bond formation.

Keywords: ribosome; rRNA; antibiotics; sparsomycin; peptidyl transferase

Introduction

Sparsomycin (Spm: Figure 1) inhibits the ribosomal peptidyl transferase (PT) reaction in all organisms studied (Vazquez, 1979; Cundliffe, 1981). This suggests that the drug recognizes a universally conserved, functionally important site in the ribosomal peptidyl transferase center. Early studies showed that Spm interferes with the binding of tRNA to the ribosomal A-site while enhancing affinity of the P-site for peptidyl-tRNA (Vazquez, 1979). However, in spite of wide use of Spm in laboratory practice and the interest in it as a potential anti-cancer compound, the details of its action are unclear.

rRNA plays an important role in ribosomal function (Noller, 1984). Thus, 23 rRNA assists in the proper placement of substrates of the peptidyl transferase reaction (Samaha et al., 1995) and may even be involved in catalysis of the peptide bond formation (Noller et al., 1992). Many known inhibitors of peptidyl transferase exert their effects by directly interacting with 23 S rRNA, as was revealed by protection of rRNA bases from chemical modification by antibiotics and the existence of rRNA mutations conferring drug resistance (Cundliffe, 1990). The mode of interaction of Spm with the ribosome is, however, more obscure. In particular, it is unclear if Spm inhibition of PT is mediated by interaction of the drug with rRNA. Studies of in vivo and in vitro activity of Spm derivatives showed that hydrophobic interactions play an important role in drug binding, suggesting that ribosomal proteins may be primarily involved in formation of the drug-binding site (Lázaro et al., 1991a). Furthermore, in contrast to many other antibiotics, Spm does not produce clear footprints on rRNA (Moazed & Noller, 1991). The nature of the Spm-binding site and its location on the ribosome remain largely unknown.

As a first step towards identifying a possible Spm-binding site on the ribosome, we isolated a resistance mutant of the halophilic archaeon Halobacterium halobium. Since H. halobium possesses a single set of rRNA genes (Hofman et al., 1979; Mankin & Kagramanova, 1986), the selection of

Abbreviations used: Spm, sparsomycin; Ths, thiostrepton; MIC, minimal inhibitory concentration; PT, peptidyl transferase.
mutations in the chromosomal rRNA genes is as probable as selection of mutations in the ribosomal protein genes. The isolated Spm-resistant mutant contained a single nucleotide substitution in the 23 S rRNA. Characterization of the Spm-resistant mutation in the peptidyl transferase center of halobacterial 23 S rRNA has allowed us to locate, for the first time, the site of rRNA essential for Spm action, revealing a possible site of interaction of this universal inhibitor of translation with the ribosome. Our findings demonstrate that rRNA plays an important role in the binding of even the antibiotics that do not produce rRNA footprints.

Results

Isolation of Spm' mutant

The archaean H. halobium is sensitive to Spm similar to all other archaean, bacterial and eukaryotic organisms tested thus far (Mankin & Garrett, 1991). The growth of H. halobium is significantly reduced at a Spm concentration of 1 µg/ml and complete inhibition is obtained at 2 µg/ml of the drug (Figure 2). In order to isolate Spm' mutant(s), H. halobium cells were grown for approximately 60 generations in the presence of 1 µg/ml of Spm followed by plating cells onto agar medium containing 2 µg/ml of Spm. A single colony appeared on the plate after two weeks incubation. As expected, a culture derived from the colony was notably more resistant to Spm when compared with the original wild-type cells (Figure 2).

Sensitivity of ribosomes from mutant cells to sparsomycin

In order to assess the Spm sensitivity of ribosomes from mutant cells, we investigated the effect of the drug on the PT reaction catalyzed by the ribosomes in vitro and on the P-site binding of peptidyl-tRNA.

In the presence of 33% (v/v) methanol, H. halobium ribosomes efficiently catalyze the PT reaction between the E. coli fMet-tRNA<sup>Met</sup> and puromycin (Monro & Marcker, 1967). The PT reaction catalyzed in vitro by wild-type H. halobium ribosomes is inhibited by Spm. However, the PT activity of the ribosomes from Spm' cells was less sensitive to Spm as compared with wild-type ribosomes (Figure 3). It should be noted that seemingly small differences in the in vitro Spm sensitivity of the wild-type and mutant ribosomes can easily account for a more pronounced difference in the cell sensitivity to the drug (Sigmund & Morgan, 1982).

In the absence of methanol, halobacterial ribosomes can still bind eubacterial fMet-tRNA<sup>Met</sup>, albeit with a very low affinity. The bound fMet-tRNA can react with puromycin, indicating that it is bound in the ribosomal P-site (data not shown). Spm is known to increase affinity of the peptidyl-tRNA to the P-site (Vazquez, 1979). The effect of Spm on binding of E. coli [³H]fMet-tRNA to wild-type and mutant H. halobium ribosomes was compared using a filter binding assay. Wild-type and mutant ribosomes bound fMet-tRNA with a similarly low affinity in the absence of Spm. However, Spm stimulated the binding of aminoacyl-tRNA significantly more strongly to the wild-type ribosomes than to the mutant ribosome (Figure 4).

Thus, the in vitro tests indicated that Spm' phenotype was linked to alterations in the ribosome.

Mapping the Spm' mutation

H. halobium contains only one set of rRNA genes; accordingly, if a mutation occurs in the 23 S rRNA gene, it will appear in every 23 S rRNA molecule. This permits the determination of the position of a mutation by direct rRNA sequencing without isolating and sequencing the rRNA gene. Since Spm is presumed to interact with the catalytic PT center,
Figure 3. Effect of Spm on peptidyl transferase reaction catalyzed by ribosomes isolated from wild-type (●) and Spm-resistant mutant (○) cells. The reaction was performed in the presence of 33% methanol (Monro & Marcker, 1967) using [H3]formylmethionyl-tRNA as a donor substrate and puromycin as an acceptor. PT activity of the wild-type ribosome in the absence of Spm was taken as 1. The Figure represents an average of three independent experiments.

Figure 4. Binding of [H3]formylmethionyl-tRNA to the wild-type and mutant ribosomes in the absence of Spm (hatched bars) or in the presence of 300 μM Spm (filled bars). The amount of bound tRNA was quantified using a filter-binding technique (see Materials and Methods).

Figure 5. Reverse transcriptase sequencing of the 23 S rRNA from the H. halobium wild-type and Spm-resistant mutant. The position of the mutation (C2518U) is indicated by an asterisk (*).
Figure 6. Secondary structure of the central loop of domain V of the H. halobium 23 S rRNA (Mankin & Kagramanova, 1986). The position of the Spmr mutation (C2518U transition) is shown by an arrow. Filled circles show positions whose alterations affect Spm-resistance (the size of the circle represents the apparent level of Spm-resistance of corresponding mutants). The lack of post-transcriptional modification of U2603 (marked by a filled square) was shown previously to affect the level of Spm-resistance (R. A. Garrett & J. P. Ballesta, personal communication). The open circle shows the position (A2088) whose mutation does not change cell sensitivity to Spm. Nucleotides protected from chemical modification by the P-site bound tRNA (Moazed & Noller, 1989) are circled and positions that photo-crosslink to $p$-azidopuromycin (Hall et al., 1988) are boxed.

of the pHRZH-borne rRNA operon (between the EcoRI site at position 1735 of the 23 S rRNA gene and a NotI site located 0.5 kb downstream from the rRNA operon) was replaced by a homologous segment of the chromosomal rRNA operon from Spm' cells. This eliminated the anisomycin-resistant mutation (position 2471) from pHRZH and introduced the C2518U mutation, while preserving A1159G and A1195G thiostrepton-resistance mutations. The entire EcoRI-NotI replacement fragment from the Spm' cells was sequenced to verify that it did not contain any mutation other than C2518U. The modified pHRZH plasmid was used to transform wild-type H. halobium cells. Transformants were selected on thiostrepton-containing agar plates and were not exposed to Spm during transformation and selection; therefore, appearance of the second-site Spm' mutations was highly unlikely. The presence of the C2518U mutation in transformed cells was verified by rRNA sequencing, and the Spm resistance of transformants was tested. Thiostrepton-resistance mutations alone did not affect cell resistance to Spm (not shown). However, cells that acquired these mutations together with the C2518U became Spm-resistant (Figure 7). Similar to the originally selected mutant, the transformed cells were able to grow and form colonies on plates containing 2 µg/ml of Spm. Therefore, we concluded that the presence of the C2518U transition in 23 S rRNA was sufficient to produce the Spm' phenotype.

Influence of other rRNA mutations in the peptidyl transferase center on sensitivity to sparsomycin

Several mutants of H. halobium, resistant to different PT inhibitors, were obtained previously in this laboratory. Some of these mutants, which contained different nucleotide substitutions within the central loop of domain V of the 23 S rRNA, were tested for their sensitivity to Spm (Figure 6). The
chloramphenicol-resistance mutation, A2088C (Mankin & Garrett, 1991), in the “upper-left” portion of the PT loop (Figure 6), did not affect Spm sensitivity. However, anisomycin-resistance mutations, C2471U (Hummel & Böck, 1987) and U2519C (unpublished) in the “right half” of the PT loop, increased cell tolerance to Spm (Figure 8), though C2471U and U2519C mutants did not reach the level of resistance of the selected Spm’ C2518U mutant. Thus, several different nucleotide alterations in the PT domain may affect Spm sensitivity.

Discussion

Spm’ mutations are clustered in the 23 S rRNA PT center

Spm is regarded as a classic inhibitor of the PT reaction. It is one of the most potent ribosome-targeted antibiotics active against organisms from all the three major evolutionary domains and, therefore, must interact with an evolutionary conserved ribosomal center (Lázaro et al., 1991a). Spm enhances interaction of the donor substrates (N-blocked aminoacyl-tRNA as well as N-blocked aminoacyl-tRNA 3’ terminal fragment) with the P-site of the ribosome while competitively inhibiting the binding of acceptor substrates to the A-site (Vazquez, 1979; Theocharis & Coutsogeorgopoulos, 1992). However, it remains unclear whether inhibition of peptide bond formation results from drug interference with acceptor substrate binding (Cundliffe, 1981), or is due to formation of an inert complex where the donor substrate is “locked” in the P-site (Monro et al., 1969). Kinetic measurements indicate that the actual mechanism of drug action can be a combination of these two, since Spm inhibits PT reaction by interacting with the ribosome in a two-step equilibrium (Theocharis & Coutsogeorgopoulos, 1992). In the first step, it acts as a competitive inhibitor (and might compete with A-site substrates), and in the second step, after isomerization of the ribosome-drug complex, the inhibition becomes mixed non-competitive, possibly reflecting the formation of an inactive complex.

The binding site of Spm is located on the large ribosomal subunit (Monro et al., 1969). Some antibiotics that target the PT center, for example, chloramphenicol and lincomycin, have been shown
to compete with Spm for binding to the ribosomes, suggesting that Spm interacts with the ribosome in the vicinity of the PT center (Lázaro et al., 1991b). However, in contrast to other inhibitors of the PT reaction (Moazed & Noller, 1987), Spm does not produce idiosyncratic footprints on the 23 S rRNA (Moazed & Noller, 1991), which precluded direct localization of its binding site.

In order to delineate the site of Spm action, we took advantage of the peculiar rRNA gene organization in an archaeon H. halobium. In contrast to the majority of other species, which have multiple rRNA operons, this organism contains only one set of rRNA genes (Hofman et al., 1979). Ribosomal proteins are encoded in single-copy genes in H. halobium, similar to other prokaryotes. Hence, mutants with alterations in ribosomal protein or rRNA genes can be isolated equally efficiently (Mankin, 1995). Prolonged growth of H. halobium cells in liquid culture, in the presence of a subinhibitory concentration of Spm, resulted in isolation of a mutant that exhibited a moderate level of resistance to Spm; it could grow in the presence of up to 5 µg/ml of the drug, while growth of the wild-type was completely abolished at 2 µg/ml Spm (Figure 2). The mutant cells contained a C2518 to U transition in the 23 S rRNA; artificial introduction of the same nucleotide alteration into wild-type cells rendered them Spm-resistant, demonstrating that C2518U mutation was sufficient to produce the Spm’ phenotype. Two other mutations in domain V of the 23 S rRNA, C2471U and U2519C, were found to confer low-level resistance to Spm.

The presence of the C2518U mutation decreased sensitivity of the ribosome to Spm in vitro. Both Spm inhibition of the PT reaction and Spm-dependent enhancement of aminoacyl-tRNA binding were less pronounced when the C2518U mutation was present in the 23 S rRNA (Figures 3 and 4). Nevertheless, the C2518U mutation did not significantly affect the normal function of the ribosome. In the absence of the antibiotic, the doubling time of the mutant cells was only slightly longer than that of wild-type cells (8.5 versus 7.5 hours), indicating that the activity of the mutant ribosomes was not significantly diminished. This was in agreement with the results of in vitro tests, where, in the absence of Spm, the PT activity of the mutant ribosomes was practically indistinguishable from that of the wild-type (Figure 3). Furthermore, the native rRNA structure was not significantly perturbed by the C2518U mutation, since accessibility of 23 S rRNA bases in the vicinity of the PT center to modification with dimethyl sulfate, kethoxal or carbodiimide was not notably altered (data not shown).

The three 23 S rRNA positions, C2471, C2518 and U2519, whose mutations result in the Spm’ phenotype, and U2603, whose modification pattern affects Spm sensitivity (Lázaro et al., 1996), are located distantly from each other in the rRNA primary structure, but are probably clustered by spatial folding of rRNA (Figure 6). The C2471U mutation is located next to A2470, which in E. coli (A2451) is protected from chemical modification by the aminoacyl moiety of the A and P-site bound aminoacyl-tRNA. In the three-dimensional ribosomal structure, this region should be in close proximity to the ‘‘bottom’’ of the PT loop, where protection of positions U2525, U2603 and U2604 (U2506, U2584 and U2585 in E. coli) depends on the 3’-terminal nucleotide of the P-site bound tRNA (Moazed & Noller, 1989), and where positions equivalent to halobacterial G2521 and U2523 could be photo-crosslinked to the A-site bound p-azidopluromycin (Hall et al., 1988; and see Figure 6).

The nature of the Spm-binding site

The location of Spm’ mutations provides insights into the interaction of the drug with the ribosome. Studies utilizing various Spm analogs have demonstrated a correlation between hydrophobicity of the drug and its activity in vivo and in vitro (van den Broek et al., 1989). Consequently, Lázaro et al. (1991a) proposed that interaction of the non-aromatic end of the Spm molecule with a hydrophobic site, presumably formed by ribosomal proteins in the vicinity of the PT center, is critical for its activity. Though our finding of Spm’ rRNA mutations does not rule out involvement of ribosomal proteins in drug binding, it nevertheless suggests that the rRNA component of the PT is intimately involved in interaction with the drug. This conclusion is compatible with our observation that functionally active large ribosomal subunits from Thermus aquaticus ribosomes, artificially depleted of a significant number of ribosomal proteins (Noller, 1993), still remain sensitive to Spm (unpublished results). On the basis of results of chemical modification of 23 S rRNA with hydrophobic psoralene, Garrett & Rodriguez-Fonseca (1995) suggested that rRNA segments corresponding to the bottom part of the PT loop in domain V of the 23 S rRNA may form a hydrophobic pocket. The Spm-resistance mutation C2518U as well as the neighboring position U2519, whose alteration gives weak resistance, are located immediately adjacent to the proposed hydrophobic rRNA region (Figure 6). Therefore, the hypothetical hydrophobic rRNA compartment may form a primary binding site for Spm. The only other known nucleotide alteration giving Spm-resistance corresponds to the lack of post-transcriptional modification of U2603 in H. halobium 23 S rRNA (Lázaro et al., 1996). U2603 corresponds to one of the positions that was modified by hydrophobic psoralene (Garrett & Rodriguez-Fonseca, 1995), which fits the idea that Spm may interact with a proposed hydrophobic pocket in 23 S rRNA.

Our finding that resistance to a universal protein synthesis inhibitor, Spm, may result from nucleotide alterations in the 23 S rRNA reinforces a growing notion that rRNA plays an important,
perhaps primary role in interaction of antibiotics with the ribosome (Cundliffe, 1990). Most of the ribosome-targeted drugs protect certain rRNA bases from chemical modification, implying close contact between antibiotics and rRNA. However, in the case of antibiotics such as Spm that do not produce clear footprints on RNA, the role of rRNA interaction with the drug remains obscure. The failure of some antibiotics to generate RNA footprints may stem from a lack of interaction with rRNA, from an inability to access certain parts of rRNA, or from the presence of chemical modifications.

Thus, interaction with rRNA is a general principle of interaction with rRNA. It is possible that antibiotics may still exert their effect on ribosome activity through interaction with rRNA. Thus, it is possible that interaction with rRNA is a general principle of action of protein synthesis inhibitors.

Materials and Methods

Enzymes and reagents

All enzymes used for cloning were from Promega. DNA sequencing was performed using the United States Biochemical Sequenase kit. AMV reverse transcriptase used for the RNA sequencing was from Seikagaku America. The following oligodeoxynucleotide primers were used for sequencing domain V of H. halobium 23 S rRNA: GTTCCCTCTGACTATACG, complementary to positions 2670 to 2688, CGGTCCCATATGTCCTCCTGGAG (positions 2482 to 2504), GTTCCCTCTGACTATACG (positions 2372 to 2388), GTTCCCTCTGACTATACG (positions 2187 to 2204) and CACCGTGGGACAGTGAG (positions 2372 to 2388), GATATGTGCTCTTGCGAG (positions 2482 to 2504), GATATGTGCTCTTGCGAG (positions 2187 to 2204), and CATTTGCGACCTGGAG (positions 2187 to 2204). Spm was obtained from the Drug Synthesis and Research Division. The previously constructed vector pHRZH (Mankin, 1995) was used to introduce the C2518U mutation into the wild-type chromosomal 23 S rRNA gene of H. halobium (Mankin et al., 1992; Mankin, 1995). The pHRZH vector contains a complete rRNA operon from H. halobium with two thiostrepton resistant mutations (A1159G and A1187G) and an anisomycin-resistance mutation (C2471U) in the 23 S rRNA gene. The EcoRI-NotI 2 kb fragment from pHRZH including domains IV to VI of the 23 S rRNA gene, the 5 S rRNA gene and a downstream region, was replaced by a similar fragment isolated from the chromosomal rRNA operon of the SpmR mutant of H. halobium. This eliminated the anisomycin-resistant mutation from the pHRZH plasmid and introduced instead the C2518U mutation. The resulting vector was used to transform the wild-type H. halobium cells (Cline et al., 1989). Transformants were plated onto an agar plate containing 1 μg/ml of thiostrepton. Ths' colonies appeared on the plate after ten days incubation at 37°C. Several colonies were inoculated into 3-ml liquid cultures and were grown in the presence of 1 μg/ml of Ths. Total RNA was isolated from the cells and the presence of the C2518U transition, in combination with the Ths' mutations, was verified by reverse transcriptase sequencing.

Measuring antibiotic sensitivity in liquid cultures

To test the antibiotic sensitivity of the wild-type, mutants or transformants, cells were first grown at 37°C in liquid cultures in the absence of antibiotics until A550 = 1.0. The cultures were diluted 1:1000 (v/v), 3 ml of diluted cultures was placed into individual tubes and varying amounts of Spm were added. Cultures were grown with shaking at 37°C until A550 = 0.7 to 0.9 for the culture grown without the antibiotic. At this point, the absorbance of the culture was monitored. The absorbance of the culture grown with the antibiotic was taken as 100%.

Growth of H. halobium and selection of the sparsomycin-resistant mutant

H. halobium, strain R1 (Stoeckenius & Kunau, 1968) was grown in liquid medium or on agar plates as described (Cline et al., 1989). The sparsomycin-resistant mutant was selected by inoculating 10 ml of liquid medium containing 1 μg/ml Spm with 10⁷ cells (1 A550 unit) and growing the culture at 37°C with constant shaking. When cell density was approaching A550 = 1, the culture was diluted 1:100 (v/v) with antibiotic-containing medium and growth was continued. The growth/dilution cycles were continued for a total of four weeks (approximately 60 cell generations). After four weeks, the diluted culture was plated onto the agar plate containing 1 μg/ml of Spm; the plate was incubated for two weeks at 37°C. Liquid culture was grown from a single colony that appeared on the plate; the total RNA was isolated and domain V of the 23 S rRNA was sequenced as described (Mankin & Garrett, 1991; Stern et al., 1987).

Introduction of the C2518U mutation into the wild-type H. halobium chromosomal 23 S rRNA gene

The previously constructed vector pHRZH (Mankin, 1995) was used to introduce the C2518U mutation into the wild-type chromosomal 23 S rRNA gene of H. halobium (Mankin et al., 1992; Mankin, 1995). The pHRZH vector contains a complete rRNA operon from H. halobium with two thiostrepton resistant mutations (A1159G and A1187G) and an anisomycin-resistance mutation (C2471U) in the 23 S rRNA gene. The EcoRI-NotI 2 kb fragment from pHRZH including domains IV to VI of the 23 S rRNA gene, the 5 S rRNA gene and a downstream region, was replaced by a similar fragment isolated from the chromosomal rRNA operon of the SpmR mutant of H. halobium. This eliminated the anisomycin-resistant mutation from the pHRZH plasmid and introduced instead the C2518U mutation. The resulting vector was used to transform the wild-type H. halobium cells (Cline et al., 1989). Transformants were plated onto an agar plate containing 1 μg/ml of thiostrepton. Ths' colonies appeared on the plate after ten days incubation at 37°C. Several colonies were inoculated into 3-ml liquid cultures and were grown in the presence of 1 μg/ml of Ths. Total RNA was isolated from the cells and the presence of the C2518U transition, in combination with the Ths' mutations, was verified by reverse transcriptase sequencing.

Assay for antibiotic sensitivity on agar plates

Wild-type, mutant and transformed cells were grown in liquid cultures in the absence of antibiotics until A550 = 0.8 to 1.0. The cultures were diluted 1:1000 (v/v), 3 ml of diluted cultures was placed into individual tubes and varying amounts of Spm were added. Cultures were grown with shaking at 37°C until A550 = 0.7 to 0.9 for the culture grown without the antibiotic. At this point, the absorbance of all the cultures was measured and normalized (absorbance of the culture grown without antibiotic was taken as 100%).
Plates were sealed in plastic bags and incubated for two weeks at 37°C.

Isolation of *H. halobium* ribosomes

Cells from one liter of exponentially growing wild-type or mutant *H. halobium* culture (A<sub>500</sub> = 0.6) were pelleted by centrifugation in a Beckman JA10 rotor (7000 rpm, ten minutes at 2°C). Cell pellets were resuspended in 35 ml of buffer A (20 mM Tris-HCl (pH 7.8), 0.1 M MgCl<sub>2</sub>, 5 mM β-mercaptoethanol) and passed through the French press at 18,000 psi. After the addition of 60 units of RNase-free RNase DNase (Promega), the cell lysate was incubated on ice for 25 minutes. The lysate was then centrifuged in a Beckman JA-20 rotor (15,000 rpm, ten minutes at 2°C) and the upper 30 ml of the resulting supernatant was layered over 15 ml of 30% (w/v) sucrose solution prepared in buffer A. Ribosomes were pelleted by centrifugation in a Beckman 42.1 rotor (36,000 rpm, 18 hours at 4°C), resuspended in 2 ml of buffer A, divided into portions, and frozen in liquid nitrogen.

In vitro peptidyl transferase reaction

The *in vitro* PT assay was performed by pre-heating 5 µl of buffer A containing 7 pmol of ribosomes for ten minutes at 42°C and subsequent mixing with 8 µl of 5 × PTP buffer (0.1 M Tris-HCl (pH 8.0), 0.1 M KCl, 0.1 M MgCl<sub>2</sub>, 5 µl of [³H]Met-tRNA<sup>Met</sup> (2000 cpm/µl; 40 Ci/mmol), 17 µl of water, 3 µl of 10 mM puromycin (neutralized to pH 7 with NH₄OH) and 2 µl of 5pm (various dilutions in dimethyl sulfoxide). The mixture was kept on ice and the reaction was initiated by the addition of 20 µl of ice-cold methanol (Monro & Marcker, 1967). Reactions were incubated for 30 minutes on ice; 10 µl of 10 M NaOH was then added and reaction mixtures were incubated for 20 minutes at 37°C. This was followed by addition of 200 µl of 1 M K₂HPO₄ and 1 ml of ethyl acetate. [³H]formyl-methionyl-puromycin was extracted into the ethyl acetate phase by vortexing tubes for one minute and spinning in the microcentrifuge for one minute; 0.5 ml of the ethyl acetate phase was mixed with 10 ml of scintillation cocktail and counted in a scintillation counter.

Filter binding assay

*H. halobium* 70 S ribosomes (160 pmol) were incubated for 30 minutes at 37°C with 0.8 pmol of [³H]Met-tRNA<sup>Met</sup> (specific activity 40 Ci/mmole) in 24 µl of reaction mixture containing (final) 15 mM Tris-HCl (pH 7.8), 2.55 M KCl, 75 mM MgCl<sub>2</sub> 1.5 mM β-mercaptoethanol. Spm was added to a final concentration of 300 µM when necessary. Reaction mixtures were diluted with 1 ml of cold buffer A and immediately filtered through a nitrocellulose filter (Millipore GSTF 02500, pore size 0.22 µm). Filters were subsequently washed twice with 1 ml of cold buffer A. Filters were dried and the amount of bound radioactivity was determined in a scintillation counter.

Acknowledgements

We thank M. E. Johnson for support, R. A. Garrett and J. P. Ballesta for communicating their results prior to publication, T. Tenson for helpful discussions and help in preparation of ³H-tRNA and P. Kloss for assistance in preparing the manuscript. This work was supported by a Hans Vahlteich Research Award and grant #95-05 from the Illinois Division of the American Cancer Society.

References


*Edited by D. E. Draper*