Dimethylation of Adenine and the Resistance of *Streptomyces erythraeus* to Erythromycin

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The erythromycin-producing organism, *Streptomyces erythraeus*, is highly resistant to erythromycin and also to other members of the macrolide, lincosamide and streptogramin type B (MLS) group of antibiotics. Resistance results from the presence of a single residue of N^6,N^6 -dimethyladenine in *S. erythraeus* 23S rRNA. The enzyme which produces this residue ('the erythromycin-resistance methylase') has been isolated and characterized *in vitro*.

INTRODUCTION

The macrolide antibiotic, erythromycin, is produced by *Streptomyces erythraeus* (McGuire *et al.*, 1952), an organism which is resistant to erythromycin and also to other members of the 'MLS' (macrolide, lincosamide and streptogramin B) group of antibiotics (Graham & Weisblum, 1979). The MLS-resistance phenotype was first observed in certain clinical strains of *Staphylococcus* and *Streptococcus* in which inducible resistance to these drugs was associated with the appearance of N^6,N^6 -dimethyladenine in 23S rRNA (Lai & Weisblum, 1971). Subsequently, it was shown that the ribosomes of such strains of *Staphylococcus aureus* were resistant to MLS antibiotics as a direct consequence of the presence of dimethyladenine in their 23S RNA (Lai *et al.*, 1973). More recently, the methylase responsible for this modification has been purified from *Bacillus subtilis* carrying the staphylococcal plasmid pE194 (Shivakumar & Dubnau, 1981).

Evidence that the MLS-resistance phenotype of *S. erythraeus* was a property of its ribosomes was presented by Teraoka and Tanaka (1974), who showed that they were resistant to various macrolides and also to lincomycin. This, together with the observation that the 23S RNA of *S. erythraeus* contained N^6,N^6 -dimethyladenine, led to the suggestion that the mechanism of MLS-resistance in *S. erythraeus* might be similar to that in *Staphylococcus* (Graham & Weisblum, 1979). Our interest in the strategies by which antibiotic producing organisms avoid the problem of auto-toxicity encouraged us to test this hypothesis experimentally.

In order to do this, it was first necessary to isolate the methylase responsible for the presence of dimethyladenine in *S. erythraeus* 23S rRNA. Then, the enzyme was used to modify heterologous rRNA *in vitro* so that the properties of reconstituted ribosomal particles containing specifically methylated RNA could be examined. Given the relative ease with which 50S ribosomal subunits of *Bacillus stearothermophilus* can be assembled *in vitro* (Fahnestock *et al.*, 1974), we decided to examine the effects of RNA methylation in this system.

METHODS

Growth of cells and preparation of extracts. Spores of *S. erythraeus* (NRRL 2338) were used to inoculate flasks of tryptic soy broth (Difco) and cultures were grown with shaking at 37 °C for 36 h. Cells were harvested by centrifugation at 15000 g for 5 min, followed by two cycles of resuspension in buffer containing 10 mM-Tris/HCl (pH 7.6 at 20 °C), 10 mM-MgCl₂, 50 mM-NH₄Cl and 3 mM-2-mercaptoethanol. Cell breakage was achieved by two

Abbreviations: HVPE, high-voltage paper electrophoresis; MLS, macrolide, lincosamide, streptogramin B group of antibiotics; SAH, S-adenosylhomocysteine; SAM, S-adenosyl-L-methionine.

passes through a French pressure cell (precooled to 0 °C) at 70 to 80 MPa. Deoxyribonuclease (5 µg ml⁻¹ final concentration) was added between pressings. The extract was then centrifuged at 30000 g for 20 min to obtain the 'S30' fraction.

Chemostat-grown cells of *B. stearothermophilus* (NCIB 8924) were obtained from the Microbiological Research Establishment, Porton Down, Wilts, U.K. S30 extracts were prepared as described above, except that cells were broken by grinding with alumina.

Preparation of ribosomes and ribosomal subunits. Ribosomal subunits of *B. stearothermophilus* were prepared using the Ti15 zonal rotor as described by Fahnestock *et al.* (1974). Ribosomal subunits and postribosomal supernatant (S100) from *Escherichia coli* MRE 600 were prepared by procedures described previously for *Bacillus megaterium* (Stark & Cundliffe, 1979).

Preparation of ribosomal RNA. RNA from 70S ribosomes (RNA70) or 50S subunits (RNA50) was prepared by extraction with urea plus LiCl and the RNA50 was freed of 5S rRNA and deproteinized to yield 23S rRNA as described by Fahnestock *et al.* (1974).

Assays of cell-free protein synthesis. The poly(U)-directed synthesis of polyphenylalanine was assayed as described previously (Cundliffe *et al.*, 1979). Here, 50S ribosomal subunits (native or reconstituted) were supplemented with *E. coli* 30S subunits, antibiotics (if any) and *E. coli* S100 prior to assay at 37 °C.

Preparation of 'erythromycin-resistance methylase'. All steps in this procedure were performed at 0–4 °C unless otherwise stated. Extracts of *S. erythraeus* were prepared as described above and were then centrifuged at 36000 rev. min⁻¹ for 16 h in the Beckman Ti70 rotor. The pellet of ribosomes and membranes was resuspended in buffer containing 10 mM-Tris/HCl (pH 7.6 at 20 °C), 30 mM-MgCl₂, 1 M-NH₄Cl and 3 mM-2-mercaptoethanol. After standing on ice for 5 h, the suspension was layered over an equal volume of the same buffer containing 20% (w/v) sucrose and centrifuged at 40000 rev. min⁻¹ for 16 h in the Ti70 rotor. Ammonium sulphate (23.7 g per 100 ml) was added to the resultant supernatant. The precipitate was removed by centrifugation at 30000 g for 15 min and discarded. Further ammonium sulphate (9.3 g per 100 ml) was added to the supernatant, which was then recentrifuged as above. The pellet was dissolved in TM buffer (20 mM-Tris/HCl pH 7.6 at 20 °C, 10 mM-MgCl₂, 3 mM-2-mercaptoethanol and 10% glycerol) and dialysed overnight against TM buffer containing 1 M-NH₄Cl. The enzyme preparation was then diluted tenfold with TM buffer and 0.3 g SAH-Sepharose was added. This resin was prepared by covalent linking of *S*-adenosylhomocysteine (SAH) to Sepharose (Pharmacia) via a six-carbon spacer arm, as described by Izzo & Gantt (1977). After 10 min at 20 °C, the suspension was poured into a column (0.7 × 4 cm) and washed with 4 vol. TM buffer containing 100 mM-NH₄Cl. Methylase activity was eluted with TM buffer containing 1 M-NH₄Cl and stored at –70 °C.

Assay of rRNA methylase activity. RNA methylase assays were performed at 37 °C and contained 50 mM-HEPES/KOH, pH 7.5, 15 mM-Tris/HCl (pH 7.6 at 20 °C), 7.5 mM-MgCl₂, 200 mM-NH₄Cl, 4% glycerol, 2 mM-2-mercaptoethanol, methylase (10–100 units ml⁻¹), 0.4 µM-RNA70 from *B. stearothermophilus* and *S*-adenosyl-L-[methyl-³H]methionine ([³H]SAM) [25 µCi ml⁻¹; sp. act. 500 mCi mmol⁻¹ (18.5 GBq mmol⁻¹)]. The incorporation of [³H]methyl groups into RNA was measured as previously described (Thompson & Cundliffe, 1981). One unit of enzyme was defined as the amount that catalysed the incorporation into RNA of 1 pmol methyl groups min⁻¹.

Bulk methylation and hydrolysis of RNA. Purified *B. stearothermophilus* 23S rRNA was radioactively methylated as described above. The reaction mixture was desalted on Sephadex G-25 and the RNA was deproteinized with phenol, precipitated with ethanol, and a portion hydrolysed with 1 M-HCl as described elsewhere (Cundliffe & Thompson, 1979). Alternatively, methylated RNA was hydrolysed in 1 M-KOH at 37 °C for 16 h.

RESULTS

Purification of a methylase from S. erythraeus

Extracts of *S. erythraeus* catalysed the methylation of rRNA from *B. stearothermophilus* using [³H]SAM as cofactor (Table 1). The methylase activity sedimented with the ribosomes on ultracentrifugation, but was released by the salt-washing procedure. Ammonium sulphate precipitation was then used to concentrate and further purify the enzyme. A significant increase in activity was reproducibly observed at this stage. Finally, the enzyme was bound to an affinity resin (consisting of SAH covalently linked to Sepharose), from which it was eluted by raising the salt concentration.

Identification of the methylated residue

The identity of the methylated residue(s) produced within rRNA by the partially purified enzyme was established by a combination of high-voltage paper electrophoresis (HVPE) and paper chromatography. *Bacillus stearothermophilus* 23S rRNA was radioactively methylated and hydrolysed with either alkali or acid. Alkaline hydrolysis of RNA degrades it to nucleoside 3'-

Fig. 1. Identification of methylated residue. *Bacillus stearothermophilus* 23S RNA was radioactively methylated and hydrolysed as described in Methods. An acid hydrolysate was subjected to descending paper chromatography on Whatman 3MM paper in solvent containing (a) butan-1-ol/water (86:14, v/v) or (b) propan-2-ol/water/ NH_3 (85:15:1.3, by vol.). Alternatively (c), alkali-hydrolysed methylated RNA was subjected to HVPE on Whatman 52 paper at pH 3.5 as described elsewhere (Sanger & Brownlee, 1967). Markers were detected by UV absorption: N^6 -MeA, N^6 -methyladenine; N^2 -MeA, N^2,N^6 -dimethyladenine; Up, Ap, Gp, Cp, nucleoside 3'-monophosphates.

Table 1. Purification of *S. erythraeus* rRNA methylase

Stage of purification	Protein (mg)	Enzyme activity (units)	Specific activity (units mg^{-1})	Purification factor	Yield (%)
S30	1031	8450	8.2	1.0	100
Ribosomal salt-wash	333	6450	19.4	2.4	76
$(\text{NH}_4)_2\text{SO}_4$ (40–55% saturation)	89	9950	112	14.0	118
SAH-Sepharose	5.2	7700	1480	180	91

monophosphates, except at residues of 2'-*O*-methylribose, where dinucleotides are generated (Bock, 1967). Following alkaline hydrolysis and HVPE at pH 3.5, [^3H]methyl radioactivity co-migrated exclusively with adenosine 3'-monophosphate (Fig. 1). This implied methylation of either the base or the sugar moiety of an adenosine residue; neither methylation affects the charge on the molecule. The identity of the methylated adenosine was established by paper chromatography of acid hydrolysates of methylated RNA. Acid hydrolysis generates pyrimidine nucleoside monophosphates and free purines, together with ribose and inorganic phosphate (Schmidt, 1957), and, of these, only the purine bases migrate from the origin in the chromatographic systems employed here. In both solvent systems used, radioactivity co-migrated with N^6,N^6 -dimethyladenine and this was the sole methylated residue (Fig. 1).

Effect of RNA methylation on ribosomes

The stoichiometry of methylation both of total rRNA (Fig. 2) and of purified 23S rRNA from *B. stearothermophilus* (results not shown) was about 1.5 methyl groups per (23S) RNA molecule, and all these methyl groups were recovered as dimethyladenine (Fig. 1). Given the previous report that N^6 -monomethyladenine does not occur in the 23S rRNA of *B. stearothermophilus* or other Gram-positive organisms (Tanaka & Weisblum, 1975), adenine residues (rather than

Fig. 2

Fig. 3

Fig. 2. Time courses of methylation (○) and acquisition of resistance to spiramycin (●). Methylation reactions contained 100 units methylase ml⁻¹ together with either [³H]SAM or unlabelled SAM (50 μM) as cofactor (see Methods). At intervals, samples containing 80 pmol *B. stearothermophilus* RNA70 were removed from the incubation containing unlabelled SAM, rapidly cooled to 0 °C in the presence of 0.2% (w/v) bentonite (Serva) to stop methylation and centrifuged at 12000 *g* for 1 min. Ribosomal particles were then reconstituted using RNA in the supernatant together with a 1.5-fold molar excess of *B. stearothermophilus* 50S subunit proteins as described previously (Fahnestock *et al.*, 1974). Reconstituted particles were precipitated with ethanol (33%, v/v, final), supplemented with postribosomal supernatant plus 30S subunits from *E. coli* and utilized in polyphenylalanine synthesis. In the absence of drug, 4–7 pmol phenylalanine were polymerized per pmol ribosomes in 10 min; otherwise, ribosomes were preincubated with 20 μM-spiramycin for 10 min at 37 °C and their activity over a subsequent 10 min incubation period was expressed as a percentage of the control value. This percentage value was taken as a measure of the resistance of the reconstituted particles to spiramycin. All points represent the means of duplicate determinations.

Fig. 3. Effect of methylation of rRNA on response of reconstituted 50S particles to spiramycin and lincomycin. Reconstituted 50S ribosomal subunits of *B. stearothermophilus* were prepared (as described in the legend to Fig. 2) using RNA70 which had been incubated with methylase (100 units ml⁻¹, for 20 min) in the presence (○) or absence (●) of SAM. These were then assayed for activity in polyphenylalanine synthesis in the presence of various concentrations of spiramycin (a) or lincomycin (b) as in the legend to Fig. 2. For comparison, results obtained with native 50S subunits of *S. erythraeus* (△) and *B. stearothermophilus* (▲) are included. When supplemented with 30S subunits and postribosomal supernatant from *E. coli* these polymerized, respectively, 8 and 15 pmol phenylalanine per pmol ribosomes in 10 min in the absence of drug. These results are representative of several similar experiments.

monomethyladenine) must be the substrate for the methylase. Accordingly, we conclude that after methylation about 75% of the 23S RNA molecules contained dimethyladenine.

In order to assess the functional significance of such methylation, the properties of reconstituted particles containing methylated 23S RNA were examined. In these experiments, *B. stearothermophilus* RNA was incubated with methylase plus unlabelled SAM as cofactor and was then used in the reconstitution of 50S subunits. These were tested, in conjunction with *E. coli* 30S subunits, for their response to spiramycin (a macrolide antibiotic which, unlike erythromycin, is an effective inhibitor of cell-free polyphenylalanine synthesis). Reconstituted particles were found to be 75% resistant to spiramycin when the average stoichiometry of methylation was 1.5 (Fig. 2). Moreover, the time courses of methylation and acquisition of spiramycin resistance were indistinguishable. It is not clear why 25% of the *B. stearothermophilus* 23S RNA molecules were refractory to methylation, since we have obtained stoichiometries of methylation close to 2.0 with rRNA from other sources such as *Streptomyces coelicolor* and *Streptomyces lividans* (data

not shown). Presumably, *S. erythraeus* rRNA is completely methylated *in vivo* (i.e. with stoichiometry, in this context, of 2·0), thereby accounting for the total insensitivity of its ribosomes to spiramycin (Teraoka & Tanaka, 1974; see also Fig. 3).

Definitive proof that methylation of rRNA is the cause of MLS resistance is provided in Fig. 3. Reconstituted ribosomes containing methylated RNA were substantially resistant to spiramycin and lincomycin over a wide range of concentrations. In contrast, those containing unmethylated RNA were just as sensitive as control native ribosomes.

DISCUSSION

A causal connection has been established in this study between the presence of dimethyladenine in *S. erythraeus* 23S rRNA and the resistance of its ribosomes to MLS antibiotics. As yet, we cannot rigorously exclude the possibility that factors other than RNA methylation might also contribute to the resistance of *S. erythraeus* to these drugs. However, the *S. erythraeus* methylase gene has recently been cloned into *S. lividans*, where it confers the MLS-resistance phenotype (Thompson *et al.*, 1982). Thus, it is now clear that dimethylation of a single adenine residue within 23S rRNA is a necessary and sufficient condition for rendering ribosomes (and organisms) resistant to erythromycin and other MLS antibiotics.

This is the second case in which ribosomal modification has proved to be the mechanism by which an antibiotic-producing organism defends itself against its product. In the first, the thiostrepton-producer, *Streptomyces azureus*, was found to methylate a specific ribose residue in its 23S rRNA (Cundliffe, 1978; Cundliffe & Thompson, 1979). It remains to be seen how widespread this type of target-site modification may be among other antibiotic producers; in particular, resistance mechanisms in certain streptomycetes which produce MLS antibiotics other than erythromycin might bear closer scrutiny. In these organisms, induction of resistance to MLS antibiotics has been circumstantially linked with the appearance of either mono- or dimethyladenine, or both, within 23S rRNA (Fujisawa & Weisblum, 1981). Further biochemical and genetic studies will be necessary to determine whether any causal connection exists between these events.

Clearly, it will be of interest to determine the site of action of the erythromycin-resistance methylase within 23S RNA. Assuming that methylation blocks the binding of MLS antibiotics to the ribosome, such studies should identify the region of 23S RNA present at their binding site. Granted the further assumption that antibiotics bind to components involved in those functions which they inhibit, this region of rRNA may well lie in the peptidyltransferase centre of the ribosome, since some MLS antibiotics appear to inhibit this function (for a review see Gale *et al.*, 1981).

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