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Chemical probing of a virginiamycin M-promoted conformational change of the peptidyl-transferase domain

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ABSTRACT

Previous findings suggest the location of the central loop of domain V of 23S rRNA within the peptidyltransferase domain of ribosomes. This enzymatic activity is inhibited by some antibiotics, including type A (virginiamycin M or VM) and type B (virginiamycin S or VS) synergimycins, antibiotics endowed with a synergistic action *in vivo*. In the present work, the ability of VM and VS to modify the accessibility of 23S rRNA bases within ribosomes to chemical reagents has been explored. VM afforded a protection of rRNA bases A2037, A2042, G2049 and C2050. Moreover, when ribosomes were incubated with the two virginiamycin components, the base A2062, which was protected by VS alone, became accessible to dimethyl sulphate (DMS). Modified reactivity to chemical reagents of different rRNA bases located either in the central loop of domain V or in its proximity furnishes experimental evidence for conformational ribosome alterations induced by VM binding.

INTRODUCTION

The functional role of ribosomal components, proteins and rRNAs, in different steps of protein synthesis has been largely explored by the use of antibiotics (1–3). Thus, the interaction of macrolides, lincosamides and type B streptogramins (the so called MLS_b group of antibiotics) with bacterial ribosomes has contributed to the unraveling of the topography of the peptidyltransferase domain. Within this enzymatic center of the 50S subunit, overlapping and non-overlapping antibiotic binding sites have been mapped (4).

Chemical, enzymatic and genetic approaches have been used to identify the ribosome components present within the peptidyltransferase domain (5–9). The relationship between several L proteins and two portions of the 23S rRNA, parts of domain V and II, with the catalytic center of this enzyme has been recognized (10–14). The chemical probing method devised by Noller and coworkers (15) has allowed the identification of rRNA bases involved in antibiotic binding to ribosomes: these bases are indeed protected by ribosome-bound antibiotics against the attack by nucleic acid reagents. Some of the bases protected by macrolides, type B synergimycins and chloramphenicol (A2057, 2058, 2062 and 2451, and G2505) were located within the central loop of domain V, and others (A752) belonged to domain II of 23S rRNA (16).

Synergimycins (streptogramins) contain two types of chemically unrelated components: type A (such as virginiamycin M or VM) and type B (such as virginiamycin S or VS), which have a pronounced synergistic action *in vivo* (17,18). *In vitro*, type A components strongly increase the affinity of ribosomes for type B components (19–21).

In the present work, the ability of type A (VM) synergimycins to modify the accessibility of 23S rRNA bases, in ribosomes, to chemical reagents was explored. Since we have previously shown that VM inactivates both the acceptor and donor substrate binding sites of peptidyltransferase (22), the present study is expected to contribute to the identification of the 23S rRNA bases involved in this enzymatic function. While the binding site of VS has been affinity labeled (12), and rRNA bases protected by this antibiotic have been identified (16), no data were so far provided on the binding site of VM. An additional aim of the present paper was to confirm that VM promotes a conformational change of ribosomes, which is presumably responsible for the synergistic effect of the two virginiamycin components.

MATERIALS AND METHODS

Inhibitors

Virginiamycins M (VM) and S (VS) were crystallized from fermentation products of *Streptomyces virginiae* (SKF-RIT, Rixensart, Belgium). Erythromycin (ERY), diethyl pyrocarbonate 95% (DEPC) and actinomycin D were purchased from Sigma (St Louis, MO). Dimethyl sulfate 99% (DMS) was from Janssen Chimica (Belgium). Kethoxal (2-keto-3-etoxybuteraldehyde) (KE) was from United States Biochemical Corporation (Cleveland, OH).

Buffers

CAC contained 50 mM Na cacodylate pH 7.2, 50 mM KCl, 75 mM NH_4Cl, 1 mM DTT, 0.5 mM EDTA and 10 mM $MgCl_2$. DMS stop solution contained 1 M Tris–HCl pH 7.5, 1 M 2-mercaptoethanol, 1.5 M NaOAc and 0.1 mM EDTA. KE stop solution was 0.5 M K borate pH 7.

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Ribosomes preparation

Ribosomes were prepared from *E.coli* strain MRE600 with standard methodology, as previously described (23).

Chemical modifications of rRNA *in situ*

Ribosomes (1 μM) were incubated with 5 μM antibiotic (ethanol solution), for 15 min at 37°C and kept in ice for 10 min prior to chemical modifications. Control ribosomes were incubated with the same amount of ethanol. Ribosome−antibiotic complexes in 250 μl of CAC buffer were incubated for 30 min at 37°C with one of the following chemical reagents: a) 5 μl of dimethylsulfate solution followed by 100 μl of DMS stop solution; b) 5 μl of diethyl pyrocarbonate; and c) 20 μl of ketoxal (37 mg/ml H_2O) followed by 20 μl of KE stop solution. In all cases, ribosomes were precipitated (2.5 vol ethanol), collected by centrifugation and resuspended in the appropriate buffer described below.

rRNA extraction

To ribosome suspensions in 200 ml of either 0.3 M NaOAc, 2.5 mM EDTA or 25 mM K borate, pH 7, 0.3 M NaOAc, 2.5 mM EDTA (KE-modified samples), SDS was added (0.5% final concentration). rRNA was extracted twice with phenol, with a phenol−chloroform−isoamylic alcohol mixture (25:24:1) and with chloroform, prior to precipitation (2 vol ethanol). Pellets were resuspended either in 25 mM K borate (KE-modified RNA) or in H_2O (other samples) and stored at −70°C.

Primer extension

Reference dideoxy sequencing tracks were obtained using unmodified rRNA as template. Three synthetic oligonucleotides were used as primers to sequence the central loop and the adjacent stems of domain V of 23S rRNA: a) PEP1 (5'TATCCTACA-CATCAAGGC-2102) was used for transcription of the upper left portion of loop V and the adjacent left stem; b) PEP3 (5'CTC-GTACTAGGAGCAGCC-2644) hybridized near the lower left part of loop V and annexed stem, and c) extension of PEP2 (5'CATACCCTTGGGACCTAC-2532) covered the right region of the loop and the corresponding right and upper stems (9). For transcription, 2 μg of rRNA were added to 20 ng of one of the PEP primers in 15 μl of 25 mM Tris−HCl pH 8, 50 mM KCl and 10 mM $MgCl_2$. Samples were heated 2 min at 90°C, and then incubated for 15 min at 50°C and for 5 min at room temperature. The extension mixture containing dCTP, dGTP and dTTP (200 μM of each), 5 μCi of α-^{35}S-dATP (1000 Ci/mmol), 2.5 μg of actinomycin D and 5 Units of AMV reverse transcriptase (Promega) in 25 mM Tris−HCl pH 8, 50 mM KCl and 10 mM $MgCl_2$, was added to the hybridization mixture. Dideoxy sequencing reactions contained 100 μM of one dideoxynucleotide. After incubation for 20 min at 42°C, 1 μl of a chase solution (1 mM each dNTP, 0.1 Unit AMV) was added, and incubation was continued for 20 min. Reaction was stopped by addition of 4 μl of 98% formamide, 10 mM EDTA, 0.2% bromophenol blue and 0.2% xylene cyanol. Samples were heated 2 min at 95°C before loading on a 8% sequencing gel and electrophoresis for 2 h at 2100 V. Reverse transcriptase stops at residue X because residue X−1 in the 5' → 3' sequence of the template is modified and cannot base pair. The nucleotide responsible for a stop is one position up in the dideoxy sequencing lanes from the stop itself.

RESULTS

Different chemical probes are known to react with 23S rRNA bases within ribosomal particles. In the present work, the following rRNA reagents were used: a) DMS, reacting with A in N−1, G in N−7 and C in N−3; b) DEPC, reacting with A in N−7; and c) KE, reacting with G in N−1 and N−2 (15). The reactivity of 23S rRNA bases to these reagents was checked by extension with reverse transcriptase (15). Three primers (PEP1, 2 and 3) were used to direct the polymerase reaction: they allowed the entire sequencing of the central loop of domain V (9).

Reactivity of 23S rRNA bases in the presence of virginiamycin M

The ability of virginiamycin M (VM) to protect rRNA bases within domain V against the attack by chemical probes was evaluated.

VM protected bases G2049 and C2050 against DMS (Fig. 1, track 2) and A2037 and 2042 against DEPC (Fig. 2, track 3).

In Table I, data concerning the upper left portion of domain V (primer PEP1) are summarized. No VM-promoted modification of base reactivity was observed in the rRNA segments primed by PEP2 and PEP3.

Influence of the simultaneous binding of type A and B synergimycins to ribosomes

It has been previously shown (19−21,24) that the binding of type A synergimycins strongly increases the affinity of ribosomes for type B synergimycins. Such an *in vitro* effect, mimicking the synergistic action of the two antibiotics *in vivo*, has also been demonstrated with ribosomes from VS-resistant bacteria (21). It was of interest to know whether the protection pattern observed in the presence of a mixture of VM and VS differed from those produced by either VM or VS. The ability of vernamycin B (a type B streptogramin) to protect bases A2062 and G2505 of the central loop of domain V has been previously described (16).

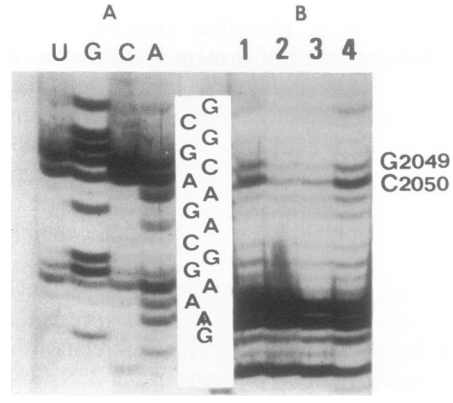

Figure 1. Protection afforded by ribosome-bound synergimycins on the attack of 23S rRNA bases by dimethylsulfate. *E.coli* ribosomes in CAC buffer were incubated with virginiamycin M (track 2), virginiamycin S (track 4), VM+VS (track 3) or no antibiotic (track 1) before incubation with DMS. Extracted 23S rRNA samples were annealed with the primer PEP1 before reverse transcription in the presence of a mixture of deoxynucleoside triphosphates containing α-^{35}S-dATP, as described in Materials and Methods. Autoradiograms of dideoxysequencing ladders (**A**) and DMS-modified (**B**) 23S rRNA are displayed.

Experiments in Figure 3 confirmed the erythromycin-mediated protection of base A2058 and A2059 (track 2) and the VS-mediated protection of A2062 (track 4) against DMS (16). However, such effect was no longer apparent when both VM and VS were present. Indeed base A2062, which was protected against DMS by VS alone (track 4), became accessible to this reagent in the presence of VM (track 5). On the contrary, the VS-mediated protection of G2505 against the attack by KE (16), was not influenced by VM (not shown).

The corresponding data, summarized in Table I, clearly indicate that VS does not modify the shielding ability of VM, whereas VM alters the protective effect of VS. In fact, the protection pattern observed in the presence of VM is similar to that occurring in the presence of a mixture of VM and VS.

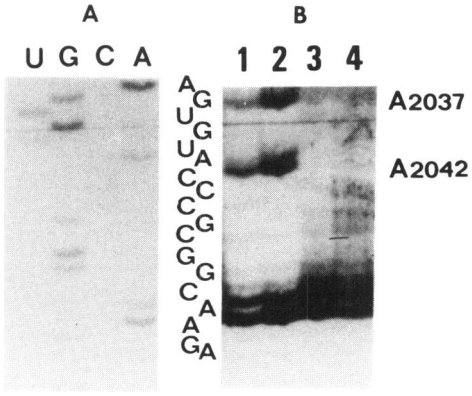

Figure 2. Antibiotic-promoted protection of 23S rRNA against diethylpyrocarbonate. *E.coli* ribosomes in CAC buffer were incubated with VM (track 3), VS (track 2), VM+VS (track 4) or no antibiotic (control, track 1) before reaction with DEPC. PEP1-primed reverse transcription of the extracted rRNA was as in the legend to Figure 1. Autoradiograms of dideoxysequencing ladders (**A**) and DEPC-modified (**B**) 23S rRNA are displayed.

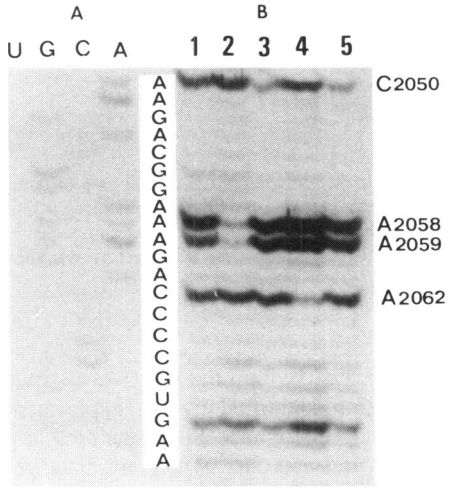

Figure 3. Virginiamycin M-induced changes of the protection afforded by virginiamycin S on A2062 against dimethylsulfate. *E.coli* ribosomes in CAC buffer were incubated with ERY (track 2), VM (track 3), VS (track 4), VM+VS (track 5) or no antibiotic (control, track 1) before submission to DMS. PEP1-primed reverse transcription of the extracted rRNA was as in the legend to Figure 1. Autoradiograms of dideoxysequencing ladders (**A**) and DMS-modified (**B**) 23S rRNA are displayed.

DISCUSSION

Comparative analysis of the reactivity of rRNA bases within control ribosomes and ribosome−VM complexes has disclosed a modified accessibility of several bases to chemical probes. Several bases (A2037, A2042, G2049 and C2050) were protected by VM. However, the reactivity of base A2062 to DMS, which was abolished in the presence of VS, was consistently and repeatedly restored when VM was simultaneously present (Table I).

Data in Figure 4 indicate that all rRNA bases, whose reactivity is modified by VM, are located outside the central loop of domain V. On the contrary, the protective effect of macrolides and type B streptogramins is restricted to bases in the central loop of domain V (encircled bases in Fig. 4) with the exception of the vernamycin B-mediated protection of base A752 in domain II (16). Such a difference is in agreement with kinetic data showing that the binding sites of VM and VS are different (4). Nevertheless, the proximity of these binding sites and those of MLS_b antibiotics to the peptidyltransferase domain is an essential prerequisite for the synergistic or competitive interactions of VM with different members of the MLS_b group (4). The ability of VM to increase ribosome affinity for VS, while decreasing that for erythromycin has been well documented (20,24).

The binding of VM to ribosomes or ribosome−VS complexes was found to produce two contrasting effects: protection of certain rRNA bases against nucleic acid reagents, and increased accessibility of other bases to such reagents. Protection could be due either to a direct shielding effect, or to steric hindrance, or to a conformational change (7). On the contrary, the increased accessibility of base A2062 seems only compatible with a conformational change of the ribosome. The involvement of such a change is best illustrated by the protection base A2062, afforded by VS alone and its accessibility to DMS in the presence of both VM and VS (Fig. 3).

The hypothesis of a conformational change has already been proposed to explain some effects produced by antibiotics: (a) VM binding is accompanied by an increased association constant of the ribosome−VS complex formation (19,20) which persists after removal of VM, but disappears after removal of some ribosomal proteins (25); (b) the ability of certain molecules to quench fluorescence of ribosome-bound VS is strongly reduced in the presence of VM (26); and (c) ribosomes from a MLS_b-resistant

Table 1. Reactivity in domain V of 23S rRNA in the presence of streptogramines A and B

23S rRNA bases[a]	Control	VM	VS	VM+VS	Chemical reagents[b]
A2037	+	−	+	−	DEPC
A2042	+	−	+	−	DEPC
G2049	+	−	+	−	DMS
C2050	+	−	+	−	DMS
A2062	+	+	−	+	DMS
G2505	+	+	−	−	KE

[a](+) = reactivity; (−) = lack of reactivity.
[b]DEPC = diethylpyrocarbonate; DMS = dimethyl sulfate; KE = kethoxal.

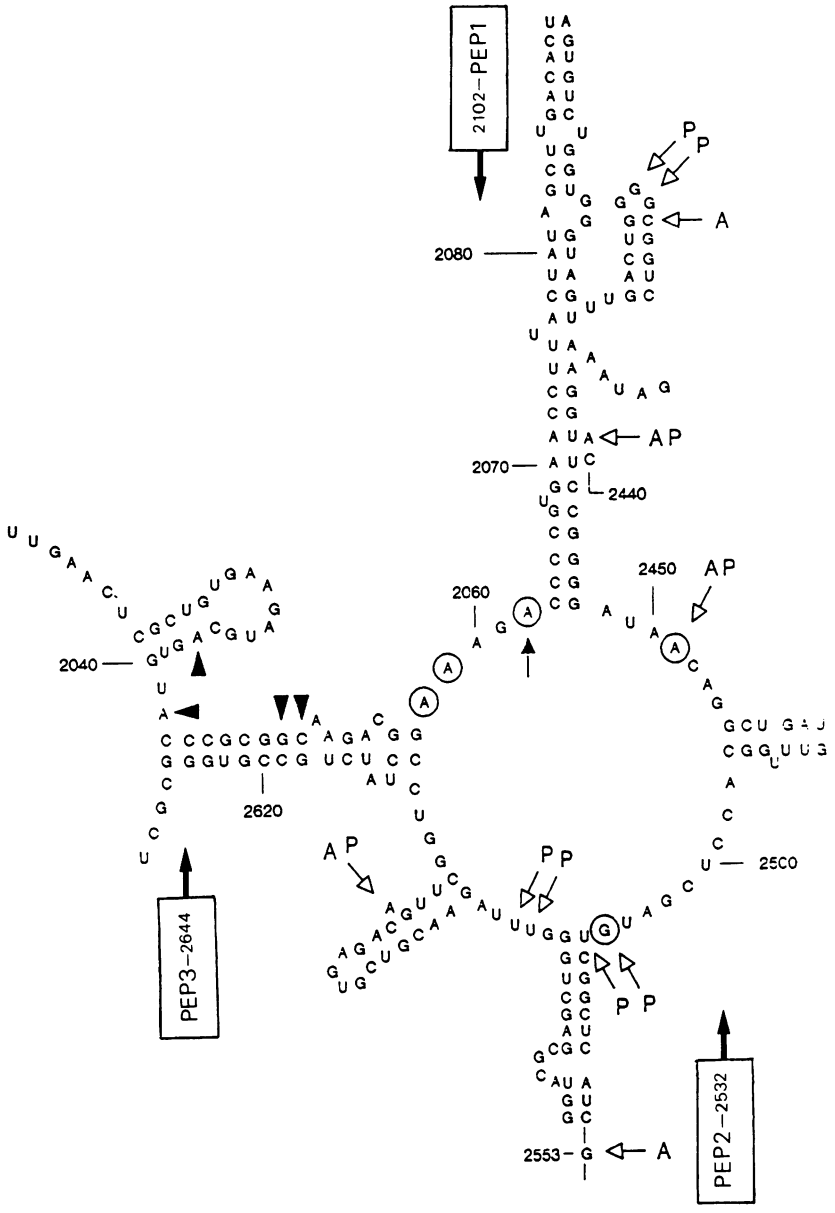

Figure 4. The protection patterns of rRNA bases induced by VM, other antibiotics and tRNA derivatives. In this drawing of the central loop of domain V of 23S rRNA and related stems, the bases displaying an altered reactivity towards chemical reagents, in the presence of ribosome-binding substances (tRNA derivatives and antibiotics) are indicated as follows: (a) bases protected by VM (▶); (b) bases protected by other antibiotics are encircled (16); (c) bases protected by aminoacyl- (A) and peptidyl- (P) tRNA (27, 28); and (d) base A2062, while protected by VS alone, became accessible in the presence of VM+VS (filled arrow). The hybridisation sites of primers PEP1, PEP2 and PEP3 and their 3'ends locations are also indicated (boxes).

strain not binding VS display high affinity VS binding in the presence of VM (21). Finally, while VM modifies the protection profile of VS, the reverse did not occur (Table I). In fact, the same rRNA bases are protected by VM, irrespective of the simultaneous presence of VS.

In conclusion, our study has furnished several lines of evidence for a VM-promoted conformational change of ribosomes, and has suggested the presumable location of such a change. The latter can account for the previously shown interference of VM with both donor and acceptor sites of peptidyltransferase (22). Related to this topic are also recent reports (27,28) indicating the protection afforded by aminoacyl or peptidyl-tRNA on several bases which are located, within the secondary structure of 23S rRNA, in the region identified by the experiments of VM-mediated protection (see reference 27 and the present work). Data from other laboratories and ours, which are displayed in Figure 4, indicate that the chemical probing technique (15), on which the present work relies, is a reliable approach to structure–function relationships of ribosomal particles.

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