INTERACTION OF SPORANGIOMYCIN WITH
THE BACTERIAL RIBOSOME

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The peptide antibiotic sporangiomycin specifically inhibits protein synthesis when added to growing cultures of *Bacillus subtilis*. A study on the reactions for bacterial protein synthesis in cell-free systems has shown that sporangiomycin interferes with specific reactions responsible for peptide-chain elongation, while it has no effect on peptide chain initiation. The primary action of the antibiotic appears to be on the 50S ribosomal subunit. This is suggested by the observation that ribosomes pre-treated with sporangiomycin are inactive in protein synthesis and that the inhibition can be overcome by an excess of 50S subunits.

Furthermore, $^{35}$S-labelled sporangiomycin binds specifically to 50S particles. Step-wise release of groups of ribosomal proteins by treatment with increasing concentrations of LiCl has shown that a specific fraction (the 1.3–1.7 M LiCl split proteins) is essential for antibiotic binding to the 50S particle. It is hypothesized that sporangiomycin inhibits protein synthesis by binding to a ribosomal multimolecular site of the utmost importance in the process of peptide-chain elongation.

Sporangiomycin is a peptide antibiotic isolated from the actinomyces *Planomonospora parontospora*. On the basis of its chemical properties sporangiomycin is related to the thiostrepton group antibiotics, which includes thiostrepton, siomycin and thiopeptin; however its chemical structure has not been conclusively determined.

The thiostrepton group antibiotics have been shown to interact specifically with the 50S ribosomal subunit of *Escherichia coli* thus preventing the binding of aminoacyl-tRNA and of EF-G factor. The results presented in this paper show that sporangiomycin inhibits protein synthesis by a similar mechanism and that $^{35}$S-labelled sporangiomycin binds specifically to the 50S subunit in a 1:1 molar ratio. Furthermore, step-wise detachment of ribosomal proteins from the 50S subunit with increasing concentrations of LiCl has shown that the split proteins contained in the 1.3–1.7 M LiCl fraction are essential for the binding of the antibiotic.

**Materials and Methods**

*E. coli* K12 (761, *his* try, L. L. Cavalli Sforza) and *Bacillus subtilis* (SB 25, *his* *try*, J. Lederberg) ribosomes and initiation factors were prepared as already described, while
polymerizing enzymes were prepared according to Conway and Lipmann\textsuperscript{11).} \textit{E. coli} EF-T elongation factor was prepared as described by Allende \textit{et al.}\textsuperscript{12) E. coli} 30S and 50S ribosomal subunits were prepared from the 1 M NH\textsubscript{4}Cl "washed" ribosomes by centrifugation in a 5~25% sucrose gradient containing 10 mM-Tris-HCl (pH 7.8), 0.5 mM Mg acetate, 50 mM NH\textsubscript{4}Cl and 6 mM \(\beta\)-mercaptoethanol. The sedimentation coefficient of ribosome-derived particles was calculated in the sucrose gradients by assuming a 50S coefficient for \textit{E. coli} ribosomal subunits prepared as described. \textit{Saccharomyces cerevisiae} (K8/6C, G. E. Magni) ribosomes and polymerizing enzymes were prepared as described\textsuperscript{13).} \textsuperscript{14C}Phenylalanine-tRNA (\textsuperscript{14C}-phe-tRNA) was prepared according to Kaji, Kaji and Novelli\textsuperscript{14)} and polyphenylalanine synthesis was assayed as described\textsuperscript{13)}, in a reaction mixture of 200 \(\mu\)l. Enzymatic and non-enzymatic binding of phe-tRNA was assayed by the procedure of Kinosita \textit{et al.}\textsuperscript{4)} in a reaction mixture of 100 \(\mu\)l. Synthesis of formyl-\textsuperscript{14C}-methionyl-tRNA (\textsuperscript{14C}-Met-tRNA) and the initiation reactions (binding of fMet-tRNA to the ribosomes and synthesis of fMet-puromycin) were performed as described\textsuperscript{10).} Sporangiomycin and \textsuperscript{35S}sporangiomycin, kindly supplied by Dr. Richard J. White of Gruppo Lepetit S.p.A., Milan, were dissolved in dioxane; the solvent did not impair the assays if its concentration in the reaction mixtures was less than 2%. Unless otherwise specified, radioactivity was determined in a Beckman liquid scintillation counter.

\textbf{Results}

\textbf{Effect of Sporangiomycin on DNA, RNA and Protein Synthesis in \textit{Bacillus subtilis}}

Sporangiomycin is active against gram-positive but not against gram-negative bacteria. Yeasts and other fungi are not affected\textsuperscript{15).} Fig. 1 shows that the addition of sporangiomycin to an actively growing culture of \textit{B. subtilis} immediately stops protein synthesis, but not RNA or DNA synthesis. These results suggest that the primary site of action of the antibiotic ought to be searched for in the machinery for protein synthesis.

\textbf{Effect of Sporangiomycin on the Reactions for Protein Synthesis \textit{in vitro}}

Sporangiomycin efficiently inhibits the reactions for peptide chain-elongation when added to cell-free systems prepared from \textit{B. subtilis} and \textit{E. coli}, while it has no effect on the systems prepared from the yeast \textit{S. cerevisiae} (Fig. 2).

These results suggest that sporangiomycin is a specific inhibitor of the procaryotic-type mechanism for protein synthesis, the "\textit{in vivo}" resistance of gram-negative bacteria being possibly due to impermeability of the cell envelopes or to the presence of an antibiotic degrading system.

Table 1 shows that the inhibition of the reactions for peptide chain-elongation by sporangiomycin can be overcome by an excess of 50S subunits, but not of 30S or supernatant enzymes. Thus the action of sporangiomycin appears to be related to the 50S subunit.

Table 2 shows that \textit{E. coli} and \textit{B. subtilis} ribosomes pretreated with sporangiomycin are inactive in protein synthesis. A possible explanation, confirmed by the experiments described below, is that ribosomes bind the antibiotic and that the complex is stable under the experimental conditions used in the assays.

An analysis of the inhibitory effect of sporangiomycin on the reaction for protein synthesis \textit{in vitro} has shown that the antibiotic interferes strongly with EF-T
Fig. 1. Effect of sporangiomycin on the incorporation of $^{14}$C-phenylalanine (A), $^{14}$C-uracil (B) and $^{14}$C-thymine (C) by intact cells of *B. subtilis*.

Time of addition of sporangiomycin is indicated by an arrow. *B. subtilis* (strain 556/1, thy, M.Polsinelli) for $^{14}$C-thymine incorporation and *B. subtilis* (ATCC 6633) for $^{14}$C-uracil and $^{14}$C-phenylalanine incorporation, were grown in 50 ml of the Von Borstell M40 medium. When cell concentration reached $10^8$ cell/ml, 5 µg/ml of $^{14}$C-phenylalanine (sp. act. 0.017 µC/pmole) or of 2-14C-uracil (sp. act. 0.017 µC/pmole) or of 2-14C-thymine (sp. act. 0.05 µC/pmole) were added. Sporangiomycin (0.35 µg/ml) was added after 10 minutes to 25 ml aliquots of the cultures. Two-ml samples were withdrawn at intervals, diluted with 2 ml of cold 10% TCA and the radioactivity of the insoluble fraction determined in a thin window counter (25% efficiency).

Fig. 2. Effect of sporangiomycin on the reactions for peptide chain elongation in cell-free extracts prepared from *E. coli*, *B. subtilis* and *S. cerevisiae*.

Assay conditions were as described under "Materials and Methods". The reaction mixtures contained: 50 mM maleate-NH$_4^+$ buffer (pH 6.6), 12 mM Mg acetate, 5 mM β-mercapto ethanol, 0.5 mM GTP, 5 mM creatine-phosphate, 25 µg/ml creatine-phosphokinase, 1 mM reduced glutathione, 50 µg/ml spermine, 50 µg/ml polypuridilic acid, 50 pmoles/ml of $^{14}$C-phenylalanine-tRNA (specific activity 265 µC/pmole) and sporangiomycin at variable concentrations. Ribosomes and polymerizing enzymes were at the following concentrations (in µg/ml), respectively: *E. coli* 1.25 and 0.8, *B. subtilis* 1.8 and 0.9; *S. cerevisiae* 0.6 and 1.2. Incubation was for 20 minutes at 30°C. Control experiments, without the antibiotic, incorporated 2.72, 2.44 and 1.12 pmoles of phenylalanine per assay in the *E. coli*, *B. subtilis* and *S. cerevisiae* systems, respectively.

Table 1. Effect of *E. coli* ribosomes or polymerizing enzymes on the inhibitory activity of sporangiomycin in peptide chain elongation

<table>
<thead>
<tr>
<th>Additions</th>
<th>Activity (%)</th>
</tr>
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<tbody>
<tr>
<td>+ sporangiomycin</td>
<td>100</td>
</tr>
<tr>
<td>+ sporangiomycin+30 S (56 µg)</td>
<td>21</td>
</tr>
<tr>
<td>+ sporangiomycin+50 S (98 µg)</td>
<td>23</td>
</tr>
<tr>
<td>+ sporangiomycin+70 S (140 µg)</td>
<td>100</td>
</tr>
<tr>
<td>+ sporangiomycin+50 S (56 µg)+50 S (98 µg)</td>
<td>89</td>
</tr>
<tr>
<td>+ sporangiomycin+polymerizing enzymes (120 µg)</td>
<td>18</td>
</tr>
</tbody>
</table>

The composition of reaction mixtures and assay conditions were as described in Fig. 2. Each assay mixture contained 140 µg of *E. coli* ribosomes and 120 µg of *E. coli* polymerizing enzymes. Additional amounts of ribosomes or polymerizing enzymes were as indicated in the Table. When present, sporangiomycin was at the concentration of 2.5 µg/ml. The complete system without additions incorporated 3.21 pmoles of phenylalanine per assay.

Table 2. Activity of *E. coli* and *B. subtilis* ribosomes pretreated with sporangiomycin

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Percent activity</th>
<th><em>E. coli</em></th>
<th><em>B. subtilis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated ribosomes</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Treated ribosomes</td>
<td>6.5</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>Treated+untreated</td>
<td>96</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

*E. coli* or *B. subtilis* ribosomes (0.5 µg/ml) were incubated with sporangiomycin (2.5 µg/ml) for 5 minutes at 30°C in a buffer containing 0.02 M Tris-HCl (pH 7.8), 0.01 M Mg acetate, 0.05 M KCl and 0.01 M β-mercapto ethanol. Ribosomes were then pelleted by ultracentrifugation and resuspended in the same buffer. Treated and/or untreated ribosomes were used at the concentration of 0.40 µg/ml. In absence of antibiotic as described in Fig. 2. The mixture containing untreated ribosomes incorporated respectively 4.5 and 5.3 pmoles phenylalanine in the systems containing untreated ribosomes from *E. coli* or *B. subtilis*,

dependent binding of phe-tRNA to *E. coli* ribosomes (Fig. 3B), although such inhibition is observed at concentrations of the antibiotic considerably higher than those shown to inhibit the reactions for poly-U dependent polyphenylalanine synthesis.
Non-enzymatic binding of phe-tRNA is partially inhibited only at high Mg\(^{2+}\) concentrations.

On the other hand, the initiation factors dependent binding of fMet-tRNA and the synthesis of fMet-puromycin, i.e. the complete set of events leading to the formation of the first peptide bond are not affected by the antibiotic (Fig. 3A). Similar findings have been obtained when using ribosomes and initiation factors prepared from *B. subtilis* (unpublished results). This also shows that the antibiotic does not interfere with the function of peptidyltransferase. At higher concentrations sporangiomycin also inhibits the ribosome dependent GTPase activity of bacterial EF-G factor\(^1\)). The above results strongly suggest that sporangiomycin specifically interacts with the bacterial ribosome in some way as to inhibit the reactions catalyzed by EF-T and EF-G factors. Similar results have been described for the chemically related antibiotics thiostrepton\(^8,9,10\), siomycin\(^8,9,17\) and thiopetin\(^9\).

**Binding of \(^{35}\)S-Sporangiomycin to Ribosomes**

A study of the mechanism by which sporangiomycin interferes both with EF-T and EF-G functions on the bacterial ribosome hopefully will shed some light on a ribosomal site of great functional importance. As a first step toward the definition of this problem it is important to determine if sporangiomycin specifically binds to ribosomes and which ribosomal component(s) is involved in such binding. To this purpose we have undertaken a study of the interactions between \(^{35}\)S-labelled sporangiomycin and ribosomes or ribosomal derived particles.

Fig. 4 shows that \(^{35}\)S-sporangiomycin binds to the 50S subunit (but not to the 30S) of the bacterial ribosome (*E. coli* and *B. subtilis*), while it does not bind to eukaryotic type ribosomes (*S. cerevisiae*).

Furthermore from these and similar experiments under saturating concentrations.
of $^{35}$S-sporangiomycin it has been calculated that the antibiotic binds to the 50S subunit in a 1:1 molar ratio (experimental results were in the range of 0.8~1.1 moles of sporangiomycin bound per mole of ribosome). A similar result has recently been reported for thiostrepton\textsuperscript{18}. Previous findings have shown that maximum inhibition of protein synthesis by siomycin and thiostrepton is obtained at antibiotic concentrations close to those of the ribosomes\textsuperscript{7,8}. Thus the data are consistent with the hypothesis that each 50S subunit is inactivated by the binding of one molecule of sporangiomycin or of related antibiotics. On the other hand, no binding site exists on the ribosome of \textit{S. cerevisiae}, which has been shown to be insensitive to sporangiomycin (Fig. 2).

An investigation on the stability of the $^{35}$S-sporangiomycin-ribosome complex was performed as described in Table 3. A convenient analytical method was provided by the observation that sporangiomycin is soluble in 10% trichloroacetic acid, while the sporangiomycin-ribosome complex is precipitated in the expected 1:1 molar ratio.

The results showed that the preformed complex was unstable to treatment with urea and Na dodecyl sulphate (SDS), known to affect largely hydrophobic bonds, while it was not affected by salts which interact with ionic bonds.

**Binding of $^{35}$S-Sporangiomycin to Ribosome-Derived Core Particles**

In order to attempt an identification of the ribosomal component(s) responsible for the binding of sporangiomycin we have studied the binding capacity of particles derived from the 50S subunit by step-wise release of specific groups of ribosomal

![Fig. 4. Binding of $^{35}$S-sporangiomycin to ribosomes from \textit{E. coli} (A and B), \textit{B. subtilis} (C) and \textit{S. cerevisiae} (D). A: 5 mM Mg\textsuperscript{++}. B,C,D: 0.5 mM Mg\textsuperscript{++}. $^{35}$S-Sporangiomycin (20 mg/ml) incubated for 15 minutes at 30°C with ribosomes (10 mg/ml) prepared from \textit{E. coli}, \textit{B. subtilis} or \textit{S. cerevisiae} in the "reconstitution buffer" containing: 10 mM Tris-HCl (pH 7.8), 20 mM MgCl\textsubscript{2}, 300 mM KCl and 6 mM $\beta$-mercaptoethanol. Total volume was 500 $\mu$l. The mixtures were then placed on the top of a 28 ml 5 to 20% linear sucrose gradient containing 10 mM Tris-HCl (pH 7.8), 50 mM NH\textsubscript{4}Cl, 6 mM $\beta$-mercaptoethanol and 0.5 mM Mg acetate (5.0 mM in experiment A) and centrifuged at 21,000 rpm for 16 hours at 4°C in an SW 25 Spinco rotor. One ml fractions were collected with an ISCO liquid scintillation counter using a toluene-triton X-100 liquid scintillator\textsuperscript{17}.]

**Table 3. Stability of the $^{35}$S-sporangiomycin-ribosome" complex to various treatments.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$^{35}$S-sporangiomycin removed by the treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>---</td>
<td>Percent of bound</td>
</tr>
<tr>
<td>0.1 M SDS</td>
<td>0</td>
</tr>
<tr>
<td>1.0 M SDS</td>
<td>0</td>
</tr>
<tr>
<td>1.0 M Urea</td>
<td>86</td>
</tr>
<tr>
<td>6.0 M Urea</td>
<td>49</td>
</tr>
<tr>
<td>1.0 M NH\textsubscript{4}Cl</td>
<td>84</td>
</tr>
<tr>
<td>3.0 M CaCl</td>
<td>0</td>
</tr>
<tr>
<td>0.5 M LiCl</td>
<td>0</td>
</tr>
<tr>
<td>1.0 M LiCl</td>
<td>0</td>
</tr>
<tr>
<td>2.0 M LiCl</td>
<td>0</td>
</tr>
</tbody>
</table>

Thirty mg of \textit{E. coli} ribosomes were incubated with a two-fold molar excess of $^{35}$S-sporangiomycin in a total volume of 7.5 ml as described in the legend to Fig. 4. Aliquots of 0.5 ml were treated for 1 hour at 4°C with the reported reagent in a buffer containing 10 mM Tris-HCl (pH 7.8), 50 mM NH\textsubscript{4}Cl, 5 mM Mg acetate and 6 mM $\beta$-mercaptoethanol. Final volume was 5 ml. Incubation was stopped by adding an equal volume of 20% TCA. $^{35}$S-Sporangiomycin bound to ribosomes was recovered on cellulose-filters while the unbound antibiotic, which is soluble in 10% TCA, was eliminated by repeated washes. Control experiments bound sporangiomycin in a 1:1 molar ratio.
proteins caused by treatment with increasing concentrations of LiCl\(^1\).

Fig. 5 shows that \(^35\)S-sporangiomycin binds to core particles obtained with treatment for 16 hours with up to 1.4 M LiCl, while no binding occurs when higher concentrations of LiCl are used. If split proteins are added back to the core particles, the binding activity is restored together with the integrity of the 50 S particles.

Fig. 6 shows that also a partial reconstitution of the particle is capable of restoring the binding activity. The fraction obtained by treatment of the 1.3 M LiCl core particle with 1.7 M LiCl, i.e. the 1.3~1.7 M LiCl split proteins, efficiently restores the binding capacity of the 1.7 M LiCl core particle.

A preliminary analysis of the 1.3~1.7 M LiCl split proteins by disc gel electrophoresis at pH 4.5 in 8 M urea\(^2\) indicated 7~8 protein bands. Further experiments are needed in order to determine which molecule(s) is(are) responsible for sporangiomycin binding.

Conclusions

The present results show that sporangiomycin inhibits protein synthesis by binding to the 50 S ribosomal subunit. Only a small fraction of the 35 different proteins present on the subunit appear to play a role in the binding of the antibiotic.

However, further work is needed in order to identify the protein(s) directly involved and to determine its function in protein synthesis. The thiostrepton group antibiotics, to which sporangiomycin is closely related, have been shown to interfere both with EF-T and EF-G activity. Thus we can hypothesize that the antibiotic
acts on a ribosomal multimolecular site playing a central role in protein synthesis. The protein(s) directly responsible for antibiotic binding might turn out to be of the utmost importance for the complex set of reactions involved in aminoacyl-tRNA binding and translocation.

The hypothesis of a multimolecular site is supported by the finding that one of the proteins required for EF-Tu and EF-G functions, recently isolated\textsuperscript{22,23}, is not involved in thiostrepton binding.

Acknowledgement


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References


