INTERACTION OF SPORANGIOMYCIN WITH THE BACTERIAL RIBOSOME

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(Received for publication August 2, 1972)

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 50 S 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 50 S subunits.

Furthermore, ^{35}S-labelled sporangiomycin binds specifically to 50 S 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 50 S 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*[1]. On the basis of its chemical properties sporangiomycin is related to the thiostrepton group antibiotics, which includes thiostrepton, siomycin and thiopeptin[1,2]; however its chemical structure has not been conclusively determined.

The thiostrepton group antibiotics have been shown to interact specifically with the 50 S ribosomal subunit of *Escherichia coli* thus preventing the binding of aminoacyl-tRNA and of EF-G factor[3,4,5,6,7,8,9]. 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 50 S subunit in a 1 : 1 molar ratio. Furthermore, step-wise detachment of ribosomal proteins from the 50 S 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 K 12 (761, *his try*, L. L. CAVALLI SFORZA) and *Bacillus subtilis* (SB 25, his_2 try_2, J. LEDERBERG) ribosomes and initiation factors were prepared as already described[10], while

polymerizing enzymes were prepared according to CONWAY and LIPMANN[11]. *E. coli* EF-T elongation factor was prepared as described by ALLENDE *et al.*[12] *E. coli* 30 S and 50 S ribosomal subunits were prepared from the 1 M NH_4Cl "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_4Cl and 6 mM β-mercaptoethanol. The sedimentation coefficient of ribosome-derived particles was calculated in the sucrose gradients by assuming a 50 S coefficient for *E. coli* ribosomal subunits prepared as described. *Saccharomyces cerevisiae* (K 8/6 C, G. E. MAGNI) ribosomes and polymerizing enzymes were prepared as described[13]. ^{14}C-Phenylalanine-tRNA (^{14}C-phe-tRNA) was prepared according to KAJI, KAJI and NOVELLI[14] and polyphenylalanine synthesis was assayed as described[13], in a reaction mixture of 200 μl. Enzymatic and non-enzymatic binding of phe-tRNA was assayed by the procedure of KINOSHITA *et al.*[4] in a reaction mixture of 100 μl. Synthesis of formyl-^{14}C-methionyl-tRNA (f-^{14}C-Met-tRNA) and the initiation reactions (binding of fMet-tRNA to the ribosomes and synthesis of fMet-puromycin) were performed as described[10]. Sporangiomycin and ^{35}S-sporangiomycin, kindly supplied by Dr. RICHARD J. WHITE of Gruppo Lepetit S.p.A., Milan, were dissolved in dioxane; the solvent did not impare 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.

Results

Effect of Sporangiomycin on DNA, RNA and Protein Synthesis in *Bacillus subtilis*

Sporangiomycin is active against gram-positive but not against gram-negative bacteria. Yeasts and other fungi are not affected[1]. Fig. 1 shows that the addition of sporangiomycin to an actively growing culture of *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.

Effect of Sporangiomycin on the Reactions for Protein Synthesis *in vitro*

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

These results suggest that sporangiomycin is a specific inhibitor of the procaryotic-type mechanism for protein synthesis, the "*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 50 S subunits, but not of 30 S or supernatant enzymes. Thus the action of sporangiomycin appears to be related to the 50 S subunit.

Table 2 shows that *E. coli* and *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 *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/μmole) or of 2-^{14}C-uracil (sp. act. 0.017 μC/μmole) or of 2-^{14}C-thymine (sp. act. 0.05 μC/μmole) 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).

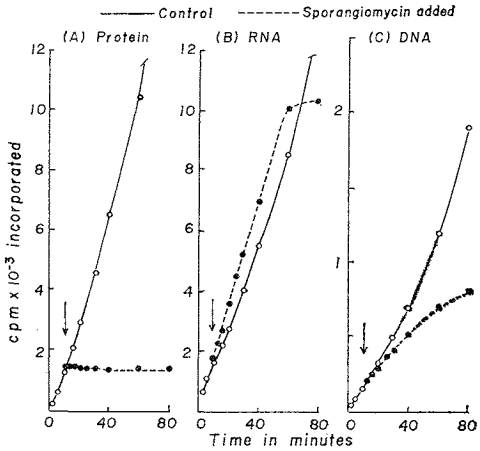


Fig. 2. Effect of sporangiomycin on the reactions for peptide chain elongation in cell-free extracts prepared from *E. coli*, *B. subtilis* and *S. cerevisiae*.

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, 20 mM NH_4Cl, 5 mM β mercaptoethanol, 0.5 mM GTP, 5 mM creatine-phosphate, 25 μg/ml creatine-phosphokinase, 1 mM reduced glutathione, 50 μg/ml spermine, 50 μg/ml polyuridilic acid, 50 pmoles/ml of ^{14}C-phenylalanine-tRNA (specific activity 265 μC/μmole) and sporangiomycin at variable concentrations. Ribosomes and polymerizing enzymes were at the following concentrations (in mg/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℃. 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.

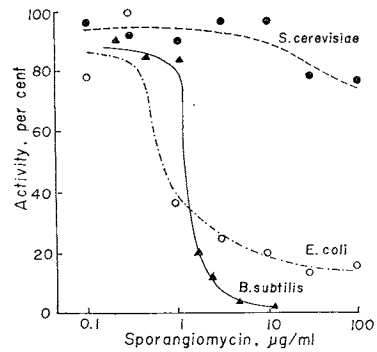


Table 1. Effect of *E. coli* ribosomes or polymerizing enzymes on the inhibitory activity of sporangiomycin in peptide chain elongation

Additions	Activity (%)
—	100
+ sporangiomycin	21
+ sporangiomycin+30 S (56 μg)	23
+ sporangiomycin+50 S (98 μg)	100
+ sporangiomycin+70 S (140 μg)	89
+ sporangiomycin+30 S (56 μg)+50 S (98 μg)	98
+ sporangiomycin+polymerizing enzymes (120 μg)	18

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

Experimental conditions	Percent activity	
	E. coli	*B. subtilis*
Untreated ribosomes	100	100
Treated ribosomes	6.5	3.5
Treated+untreated ribosomes	96	100

E. coli or *B. subtilis* ribosomes (0.5 μg/ml) were incubated with sporangiomycin (2.5 μg/ml) for 5 minutes at 30℃ 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-mercaptoethanol.

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 mg/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. 3 B), 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^{++} concentrations.

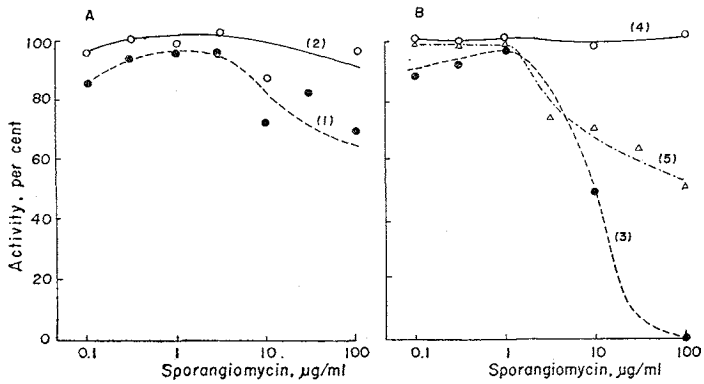
On the other hand, the initiation factors dependent binding of fMet-tRNA and the synthesis of fMet-puromycin, *i.e.* the complete set of events leading to the formation of the first peptide bond are not affected by the antibiotic (Fig. 3 A). Similar findings have been obtained when using ribosomes and initiation factors prepared from *B. subtilis* (unpublished results). This also shows that the antibiotic does not interfere with the function of peptidyl-transferase. At higher concentrations sporangiomycin also inhibits the ribosome dependent GTPase activity of bacterial EF-G factor[15]. 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[3,5,8,9,16], siomycin[3,6,7] and thiopeptin[4].

Fig. 3. Effect of sporangiomycin on the reactions for peptide chain initiation and on the binding of phenylalanine-tRNA to *E. coli* ribosomes.

A : binding of fMet-tRNA (1) and synthesis of fMet-puromycin (2). B : EF-T dependent binding of phe-tRNA (3) and non enzymatic binding of phe-tRNA at 7 mM-Mg acetate (4) and at 20 mM Mg acetate (5).

Assay conditions were as described under "Materials and Methods". The 100 μl-reaction mixture for binding of fMet-tRNA and synthesis of fMet-puromycin contained : 100 mM Tris-HCl (pH 7.2), 5 mM Mg acetate, 50 mM KCl, 5 mM β mercaptoethanol, 0.5 mM GTP, 0.8 absorbance units/ml ApUpG, 422 $p$moles/ml f-^{14}C-Met-tRNA (specific activity 233 μmole), 0.9 mg/ml ribosomes, 0.41 mg/ml initiation factors.

Incubation was for 15 minutes at 24℃. The 100 μl reaction mixture for the assay of binding of phe-tRNA contained : 50 mM Tris-HCl (pH 7.8); 7 mM or 20 mM Mg acetate ; 160 mM NH_4Cl ; 2 mM dithiothreitol ; 0.2 mM GTP ; 50 μg/ml polyuridilic acid ; 100 $p$moles/ml ^{14}C-phenylalanine-tRNA (specific activity 265 μC/μmole); 1.25 mg/ml ribosomes ; 90 μg/ml of *E. coli* EF-T factor were present when enzymatic binding was assayed. Incubation was for 10 minutes at 30℃. Data for enzymatic binding were obtained by subtracting the values for non enzymatic binding from values obtained in presence of EF-T-factor. In every case sporangiomycin was present as indicated in the figures. Control experiments, without the antibiotic were (per assay) : 2.00 $p$moles of fMet-tRNA bound ; 8.38 $p$moles of fMet-puromycin synthesized ; 3.20 $p$moles phe-tRNA enzymatically bound ; 1.56 and 4.36 $p$moles of phe-tRNA non-enzymatically bound at 7 mM and 20 mM Mg acetate, respectively.



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.

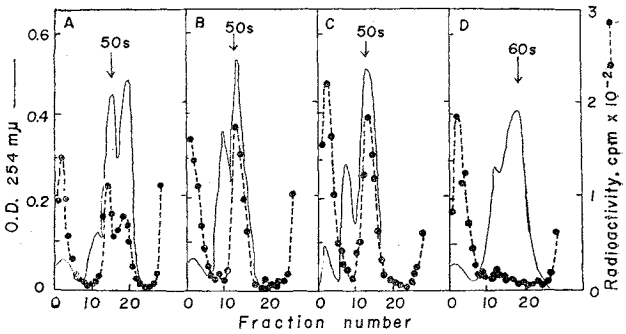
Fig. 4 shows that ^{35}S-sporangiomycin binds to the 50 S subunit (but not to the 30S) of the bacterial ribosome (*E. coli* and *B. subtilis*), while it does not bind to eukaryotic type ribosomes (*S. cerevisiae*).

Furthermore from these and similar experiments under saturating concentrations

of ^{35}S-sporangiomycin it has been calculated that the antibiotic binds to the 50 S 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[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[7,8]. Thus the data are consistent with the hypothesis that each 50 S 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 *S. cerevisiae*, which has been shown to be insensitive to sporangiomycin (Fig. 2).

Fig. 4. Binding of ^{35}S-sporangiomycin to ribosomes from *E. coli* (A and B), *B. subtilis* (C) and *S. cerevisiae* (D). A : 5 mM Mg^{++}. B,C,D : 0.5 mM Mg^{++}.

^{35}S-Sporangiomycin (20 μg/ml) incubated for 15 minutes at 30 ℃ with ribosomes (10 mg/ml) prepared from *E. coli*, *B. subtilis* or *S. cerevisiae* in the "reconstitution buffer" containing : 10 mM Tris-HCl (pH 7.8), 20 mM $MgCl_2$ 300 mM KCl and 6 mM β mercaptoethanol. Total volume was 500 μl. The mixtures were then placed on the top of a 28 ml 5~20 % linear sucrose gradient containing 10 mM Tris-HCl (pH 7.8), 50 mM NH_4Cl, 6 mM β-mercaptoethanol and 0.5 mM Mg acetate (5.0 mM in experiment A) and centrifuged at 21.000 rpm for 16 hours at 4℃ in an SW 25 Spinco rotor. One ml fractions were collected with an ISCO collector equipped with an automatic O.D.$_{254\,m\mu}$ recorder and radioactivity was determined in a Beckman liquid scintillator counter using a toluene-triton X-100 liquid scintillant[17].



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.

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

Treatment	Percent of bound ^{35}S-sporangiomycin removed by the treatment
—	0
0.1 M SDS	0
1.0 M SDS	86
1.0 M Urea	49
6.0 M Urea	84
1.0 M NH_4Cl	0
3.0 M CsCl	0
0.5 M LiCl	0
1.0 M LiCl	0
2.0 M LiCl	0

Thirty mg of *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℃ with the reported reagent in a buffer containing 10 mM Tris-HCl (pH 7.8), 50 mM NH_4Cl, 5 mM Mg acetate and 6 mM β 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.

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 50 S subunit by step-wise release of specific groups of ribosomal

proteins caused by treatment with increasing concentrations of LiCl[19].

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. 5. Binding of ^{35}S-sporangiomycin to *core particles* obtained by treatment of *E. coli* 50 S ribosomal subunits with increasing concentration of LiCl.

Ribosomal *core particles* were obtained as described by ITOH *et al.*[19] by treating 1 mg aliquots of 50 S ribosomal subunits with variable concentrations of LiCl (experiments: B, 0.3 M; C, 1.40 M; D, 2.10 M; E, 2.75 M) in a buffer containing 30 mM Tris-HCl (pH 7.8) and 100 mM Mg acetate in a final volume of 1 ml. After incubation for 16 hours at 4°C *core particles* were recovered by centrifuging 6 hours at 220.000×*g*. The pellet was assayed for its capacity to bind ^{35}S-sporangiomycin as described in the legend to Fig. 4.

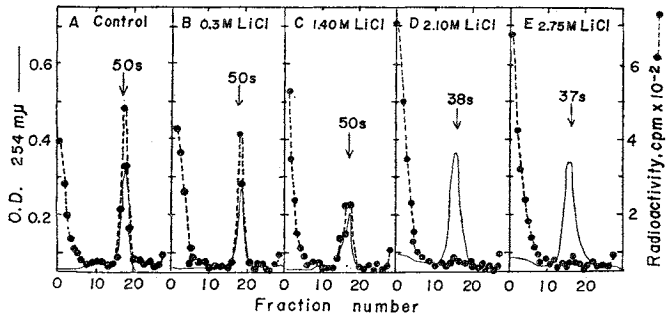


Fig. 6 shows that also a partial reconstitution of the particle is capable of restoring the binding activity. The fraction obtained by treatment of the 1.3 M LiCl *core particle* with 1.7 M LiCl, *i.e.* the 1.3~1.7 M LiCl *split proteins*, efficiently restores the binding capacity of the 1.7 M LiCl *core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

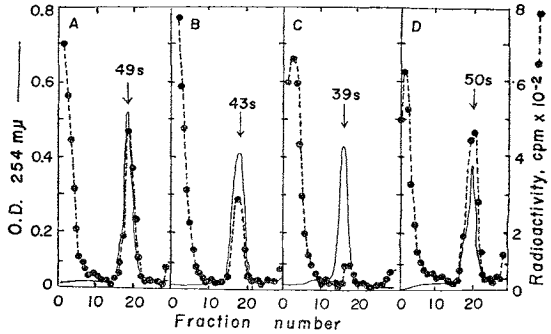
A preliminary analysis of the 1.3 ~1.7 M LiCl *split proteins* by disc gel electrophoresis at pH 4.5 in 8 M urea[21] indicated 7~8 protein bands. Further experiments are needed in order to determine which molecule(s) is(are) responsible for sporangiomycin binding.

Fig. 6. Binding of ^{35}S-sporangiomycin to partially reconstituted 50 S *E. coli* ribosomal subunits.

A: 1.7 M LiCl *core particles* +0~1.3 M LiCl and 1.3~1.7 M LiCl *split proteins*. B: 1.7 M LiCl *core particles* +1.3~1.7 ML iCl *split proteins*. C: 1.7 M LiCl *core particles*. D: 50 S subunit (control).

Ribosomal *core particles* and *split proteins* were obtained by step-wise treating 50 S ribosomal subunits with 1.3 M LiCl for 16 hours as described in the legend to Fig. 5. Pelleted *core particles* were further treated with 1.7 M LiCl for 16 hours. The 1.7 M LiCl *core particles* were resuspended in the "reconstitution buffer" (as described in Fig. 4) and the 0~1.3 M LiCl and 1.3~1.7 M LiCl *split proteins* were dialyzed for 20 hours against the same buffer.

50 S Ribosomal particles were partially or totally reconstituted by combining the 1.7 M LiCl *core particles* with both split protein fractions, or with the 1.3~1.7 M LiCl fraction, as described by NOMURA and ERDMANN[20]. The particles were then assayed for their capacity to bind ^{35}S-sporangiomycin as described in the legend to Fig. 4.



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[22,23], is not involved in thiostrepton binding.

Acknowledgement

One of us (GIORGIO PIRALI) hold a post-doctoral fellowship of the "Ciclo biennale di ricerche per la specializzazione in Genetica e Biochimica applicate alla Microbiologia Industriale" sponsored by C. Erba S.p.A., Farmitalia S.p.A., Italseber S.p.A., Lepetit S.p.A. and Pierrel S.p.A.

The authors are grateful to Dr. ORIO CIFERRI for critical discussion.

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