ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1975, p. 850-855

Vol. 7, No. 6
Printed in U.S.A.

Properties of the Ribosomes of Antibiotic Producers: Effects of Thiostrepton and Micrococcin on the Organisms Which Produce Them

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Received for publication 24 January 1975

Ribosomes of *Streptomyces azureus*, which produces thiostrepton, are resistant to thiostrepton by virtue of being unable to bind the antibiotic. These ribosomes are also resistant to a number of other antibiotics (the thiostrepton group) which may share a common ribosomal binding site and a common mode of action with thiostrepton. Conversely, *Bacillus pumilis* and a strain of micrococcus, which produce the (probably identical) antibiotics micrococcin P and micrococcin, respectively, possess ribosomes which are susceptible to these antibiotics in vitro, although the organisms themselves are resistant.

Various authors have considered at some length the natural functions (if any) of antibiotics. Under certain circumstances (always presupposing that a given antibiotic producer is insensitive to its product) production of an antibiotic might remove competitors from an ecological niche; however, it has also been argued that the advantages to be gained from secondary metabolism may be more obscure (4, 11). Thus (4), secondary metabolism might allow a cell to maintain essential enzymes and entire uptake or synthetic systems in working order after the cessation of primary metabolism (i.e., cell growth), perhaps allowing a more rapid resumption of growth if, and when, conditions became more favorable. Alternatively (11) one can consider the advantages to a cell (but not necessarily at the expense of its neighbors) of excreting secondary metabolites of no use to itself. These could arise from the action of enzymes upon normal metabolic intermediates after their accumulation in abnormal amounts after inhibition of growth. According to this hypothesis, biological activity of excreted secondary metabolites might be accidental, but not so surprising, since many of them would resemble normal metabolic intermediates and might be potent antimetabolites. Both of these hypotheses (others are not excluded) leave open the question of whether antibiotic producers should be expected to be susceptible or resistant to their products.

We have initiated a program of investigation into the effects of ribosome inhibitors upon the organisms which produce them. Surprisingly, although it was shown some time ago (10) that a streptomycin-producing *Streptomyces* was tolerant of streptomycin, there appear to have been few detailed examinations of the properties of the ribosomes of organisms which produce inhibitors of protein synthesis. There are, however, notable exceptions. Extracts of a *Streptomyces* which produces chloramphenicol, synthesizing polyphenylalanine in response to added poluridylic acid, were susceptible to inhibition by chloramphenicol and the ribosomes bound the drug indistinguishably from those of *Escherichia coli* (6). Conversely, protein synthesis in extracts of a *Streptomyces* which produces tetracycline was resistant to tetracycline, although the drug was still able to bind to the ribosomes (7).

Here we report that the streptomycete which makes thiostrepton possesses thiostrepton-resistant ribosomes which bind the drug very poorly. In contrast, the bacteria which produce micrococcin possess ribosomes which are susceptible to micrococcin in vitro, although whole cells of these organisms are micrococcin resistant.

MATERIALS AND METHODS

Growth of Streptomyces azureus and production of crude [^{35}S]thiostrepton. The organism was grown in fermentor medium which contained per liter: tryptone, 10 g; malt extract, 15 g; glucose, 20 g; yeast extract, 3 g; and maltose, 20 g. Fermentor agar as used for plates in experiments described in Table 1 was made by adding agar (1.5% wt/vol, final concentration) to the above medium. The organism was grown from spores in a starter culture (50 ml) for 36 h and this was then used to inoculate 500 ml of fermentor medium (at zero time). Growth was carried out at 25 C with aeration by shaking in a rotary incubator. At time 30, 48, and 80 h, 17 mCi of $Na_2{}^{35}SO_4$ (carrier free) was added and incubation was continued until 120 h whereupon the mycelia were harvested by

centrifugation. The pellet was extracted four times with chloroform, with one further exposure to the solvent overnight. The extract was then dried down at room temperature on a rotary evaporator under vacuum and taken up in dimethyl sulphoxide. As indicated below, 20 to 25% of the radioactive material in such a preparation can usually be characterized as thiostrepton. Further purification of [^{35}S]thiostrepton is described fully below.

Ribosomes. Regardless of their source, crude ribosomes were washed three times by centrifugation through a buffer containing 10 mM tris(hydroxymethyl)aminomethane (Tris)-chloride (pH 7.6), 10 mM magnesium acetate, 1.0 M NH_4Cl, and 1 mM dithiothreitol. These high-salt washed ribosomes were used in all in vitro assays described here. Cells were disrupted using a French pressure cell. The buffer in which cells were lysed contained: 10 mM Tris-chloride (pH 7.6), 10 mM magnesium acetate, 50 mM NH_4Cl, and 1 mM dithiothreitol.

Formation of ribosome-EF G-guanine nucleotide complexes. Buffer contained 10 mM Tris-Cl (pH 7.5), 20 mM magnesium acetate, 10 mM NH_4Cl, and 1 mM dithiothreitol. Incubation 1 consisted of 50 pmol of ribosomes with or without drug as indicated in Tables 2 and 3 in a total volume of 15 μl for 15 min at 0 C. Incubation 2 consisted of the following components added in a volume of 50 μl: elongation factor (EF) G from *E. coli*, 50 pmol; [*ring*-^{3}H] guanosine triphosphate (GTP) (13.3 Ci/mM), 49 pmol; sodium fusidate, 2.5 mM (final concentration). Incubation 2 was for 5 min at 0 C and was terminated by the addition of 3 ml of ice-cold buffer containing 0.2 mM fusidate. Ribosomes were collected on membrane HAWP filters (Millipore Corp., 0.45-μm pore size) which were washed four times with 3 ml of buffer containing 0.2

TABLE 1. *Susceptibilities of various microorganisms to selected antibiotics*[a]

Antibiotic	Organism				
	Streptomyces coelicolor	*Streptomyces azureus*	Su's *Micrococcus*	*Bacillus pumilis*	*Bacillus megaterium* KM
Chloramphenicol	S	S	S	S	S
Althiomycin		S	S	S	S
Fusidic acid		S	S	S	S
Thermothiocin		S	S	S	S
Thiostrepton	S	R	S	S	S
Siomycin	S	R	S	S	S
Sporangiomycin	S	R	S	S	S
Micrococcin	S	R	R	R	S
Micrococcin P	S	R	R	R	S

[a] Filter paper disks (5-mm diameter) containing antibiotics (10 to 25 μg per disk) were placed on freshly seeded lawns of microorganisms on fermentor agar plates (see Materials and Methods). These were then incubated for 24 h at 37 C (bacteria) or 48 h at 25 C (streptomycetes). Clear zones around disks indicated susceptibility (S) towards given antibiotics. Absence of zones indicated resistance (R). In each case, results were unequivocal and not dependent upon the duration of incubation.

TABLE 2. *Effects of antibiotics on the formation of ribosome-EF G-guanine nucleotide complexes in the presence of fusidic acid*[a]

Antibiotic added	Amount	Complex formation[b] (pmol of guanine nucleotide complexed/pmol of ribosomes) (% of control: normalized)		
		Ribosomes from *S. azureus*	Ribosomes from *S. coelicolor*	Ribosomes from *B. megaterium* KM
None		100	100	100
Thiostrepton	2.5 nmol	>90	18	
	250 pmol		55	<5
Siomycin	3.0 nmol	>90	16	
	150 pmol		44	
Sporangiomycin	3.5 nmol	>90	12	
	150 pmol		31	5
Micrococcin	2.0 nmol	>90	47	
	200 pmol		54	61

[a] EF G from *E. coli* was used in all these experiments.

[b] In controls, ribosomes of *S. azureus* were 17% as efficient, those from *S. coelicolor* 37% as efficient, and those from *B. megaterium* 30% as efficient as ribosomes from *E. coli* in supporting complex formation. Usually 50 pmol of *E. coli* ribosomes supported the formation of about 13 pmol of complex representing approximately 120,000 counts/min at a counting efficiency of 30% for tritium.

TABLE 3. *Effect of micrococcin on formation of ribosome-EF G-guanine nucleotide complexes with ribosomes from various sources*[a]

Additions	Amount	Complex formation (pmol of guanine nucleotide complexed/pmol of ribosomes) (normalized as % of control)		
		E. coli ribosomes	Su's *Micrococcus* ribosomes	*B. megaterium* KM ribosomes
None		100	100	100
Micrococcin	200 pmol	80	48	60
	4 nmol	43	38	50
	21 nmol	33	14	44
Thiostrepton	150 pmol	51	5	8

[a] EF G from *E. coli* was used throughout. In controls, *Micrococcus* ribosomes were 83% as efficient and *Bacillus* ribosomes 30% as efficient as *E. coli* ribosomes in supporting complex formation.

mM fusidate. Radioactivity retained on filters was estimated by liquid scintillation counting and was assumed to represent ribosome -EF G-guanine nucleotide complexes. In all cases blank estimations (lacking ribosomes) were performed and were used to correct experimental values which themselves were the means of duplicates.

Binding of [^{35}S]thiostrepton to ribosomes of E. coli. Buffer contained Tris-chloride (10 mM), pH 7.6, magnesium acetate (10 mM), NH_4Cl (50 mM), and dithiothreitol (1 mM). For incubation 1, ribosomes (50 pmol) were incubated with dimethyl sulphoxide (5 μl) or authentic nonradioactive thiostrepton (16 nmol in dimethyl sulphoxide) in a total volume of 45 μl for 15 min at room temperature. For incubation 2, 5 μl of [^{35}S]thiostrepton (Fig. 1) or purified [^{35}S]thiostrepton (Fig. 2) were added and incubation was continued for a further 15 min. Then 50 μl of buffer was added and the whole material was applied to a Sepharose 6B column (5 cm by 5.5 mm) previously equilibrated with buffer. Ribosomes are excluded by Sepharose 6B whereas free thiostrepton is not. Thirty-five fractions (each of 75 μl) were collected from each column and their radioactivity was estimated by liquid scintillation counting.

Antibiotics. The sources of the various antibiotics used here were: the Squibb Institute for Medical Research, Princeton, N.J. (thiostrepton); Gruppo Lepetit, Milano (thermothiocin and sporangiomycin); K. Tanaka, Shionogi & Co. Ltd., Osaka (siomycin); N. Heatley, Pathology, Univ. of Oxford (micrococcin); and J. Walker, National Institute for Medical Research (micrococcin P). These latter two antibiotics are believed to be identical (J. Walker, personal communication) and behaved indistinguishably in all assays described here.

Organisms. These were obtained as follows: *S. azureus* ATCC 14,921 from E. Meyers, Squibb Institute for Medical Research, Princeton, N.J.; Su's *Micrococcus* NCTC 7218 from N. Heatley, Pathology, Univ. of Oxford; *Bacillus pumilis* strain (micrococcin P producer) from J. Walker, National Institute for Medical Research, Mill Hill, London; *Streptomyces coelicolor* A3(2) from D. Hopwood, John Innes Institute, Norwich.

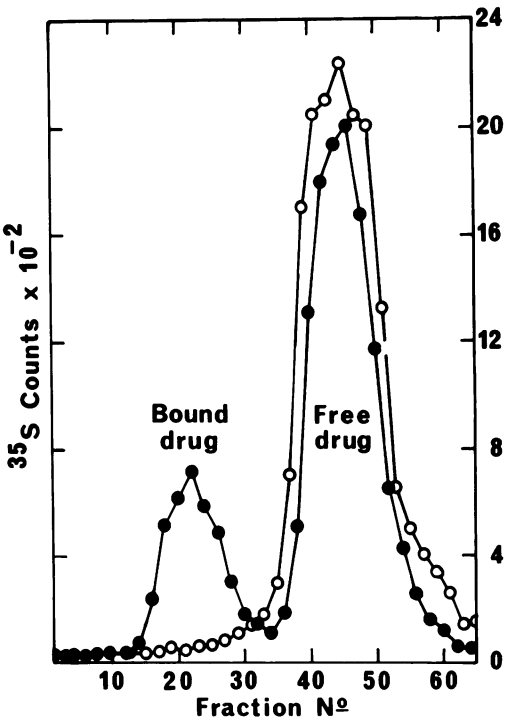

FIG. 1. *Binding of crude [^{35}S]thiostrepton to ribosomes of E. coli. Symbols: ●, binding carried out in absence of nonradioactive thiostrepton; ○, binding carried out after exposure of ribosomes to nonradioactive thiostrepton.*

RESULTS

A group of antibiotics comprising thiostrepton, siomycin, micrococcin (and the identical compound, micrococcin P), sporangiomycin, and thiopeptin have been shown to possess closely related modes of action upon bacterial protein synthesis in vivo and in vitro (E. Cundliffe and P. Dixon, manuscript in preparation).

We also determined that siomycin, thiopeptin, sporangiomycin, and, to a much lesser extent, micrococcin inhibit the binding of [^{35}S]thiostrepton to bacterial ribosomes, indicating that the binding sites for these compounds are either identical or are intimately associated on the ribosome. Accordingly, we examined the susceptibilities towards these various antibiotics of *S. azureus* which produces thiostrepton, Su's *Micrococcus* (5, 8) which produces micrococcin, and *B. pumilis* which produces micrococcin P (3). The results are given in Table 1. Although susceptible to a range of compounds which inactivate 70*S* ribosomes, *S. azureus* was resistant to the entire thiostrepton group, whereas *S. coelicolor* (included as a control to establish the fact that streptomycetes in general are not thiostrepton resistant) was susceptible to each drug employed. Cross resistance to the entire thiostrepton group was also observed in two spontaneously arising mutants of *Bacillus megaterium* KM selected for resistance to thiostrepton (P. D. Dixon, unpublished data), whereas the wild type was susceptible to these drugs. In contrast, Su's *Micrococcus* and *B. pumilis* were resistant only to the antibiotic which they produce and were susceptible to other members of the thiostrepton group.

To examine in vitro the ribosomes of these various organisms, we studied in some detail one of the partial reactions of protein synthesis. The cycle of events which accompanies the addition of each amino acid to a nascent peptide chain includes the formation of a complex between the ribosome, the protein EF G, and GTP, as a consequence of which GTP is cleaved to guanosine diphosphate and inorganic phosphate. The antibiotic fusidic acid allows the GTPase event to occur but stabilizes the resultant ribosome-EF G-guanosine diphosphate complex which can then be recovered on a membrane filter (Millipore Corp.) (2). The formation of such complexes (from either GTP or guanosine diphosphate) was specifically inhibited by the thiostrepton group of antibiotics (no other antibiotics have yet been shown to inhibit this reaction) and we took advantage of this fact to examine the susceptibilities of the ribosomes of *S. azureus* and Su's *Micrococcus* towards this group of antibiotics. Results obtained with ribosomes of *B. pumilis* were similar to those of the *Micrococcus* and are not given here. One further point which must be emphasized is that the lack of purified EF G from organisms other than *E. coli* necessitated the use of heterologous systems involving this material and ribosomes from other organisms. The efficiencies of such systems are indicated in the legends to Tables 2 and 3.

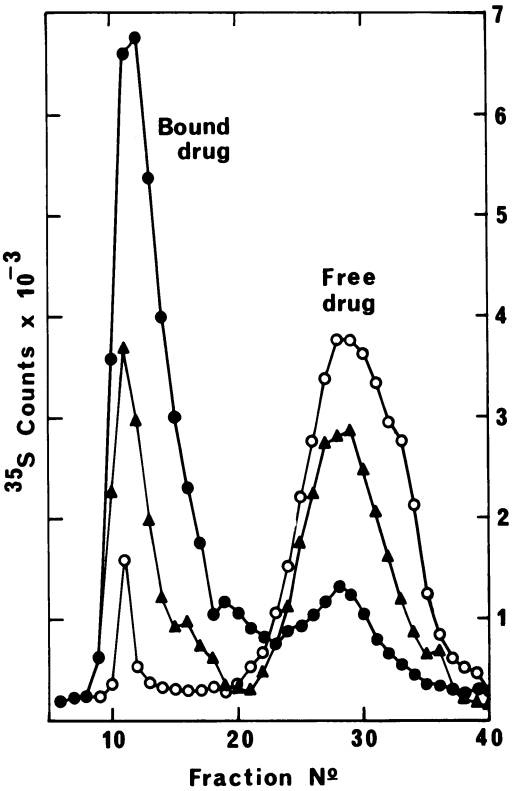

FIG. 2. *Binding of purified [^{35}S]thiostrepton to ribosomes of E. coli, S. azureus, and S. coelicolor. Each assay utilized 5 μl of purified [^{35}S]thiostrepton equivalent to 260 pmol at 40 counts/min per pmol. Symbols: ●, binding to 740 pmol of E. coli ribosomes; ○, binding to 140 pmol of S. azureus ribosomes; ▲, binding to 120 pmol of S. coelicolor ribosomes.*

As shown in Table 2, ribosomes from *S. azureus* continued to support the formation of ribosome-EF G-guanine nucleotide complexes after exposure to antibiotics of the thiostrepton group, whereas similar systems employing ribosomes from *S. coelicolor* or *B. megaterium* KM were markedly inhibited. In contrast (Table 3), ribosomes from Su's *Micrococcus* were more susceptible to micrococcin than were ribosomes from *B. megaterium* KM or *E. coli* and were, as expected from Table 1, also susceptible to other members of the thiostrepton group.

Preparation of affinity-purified [^{35}S]thiostrepton. A crude chloroform extract of the mycelium of *S. azureus*, grown with [^{35}S]sulphate as described, usually contained [^{35}S]thiostrepton to the extent of 20 to 25% of the total radioactive material present. This can readily

be estimated from the ability of this radioactive material to bind to an excess of *E. coli* ribosomes (Fig. 1). It is also apparent that all of the radioactive material which binds to ribosomes is thiostrepton, since binding can be totally abolished by prior exposure of ribosomes to authentic, nonradioactive drug. Such crude [^{35}S]thiostrepton preparations were purified by collecting ribosomes with bound drug after elution from a Sepharose 6B column (total bed volume 10 ml) and extracting them twice with 10 volumes of chloroform by prolonged shaking on a Griffin flask shaker. The resultant extract was dried down on a rotary evaporator under vacuum at room temperature and taken up in dimethyl sulphoxide. This pure [^{35}S]thiostrepton was used in the studies described here and some characteristics of its ribosomal binding are given in Fig. 2. A maximum of 75 to 80% of the radioactive material, prepared on separate occasions, could be recovered on ribosomes after chromatography on Sepharose 6B and, in experiments similar to those described in Fig. 1, prior exposure of ribosomes to nonradioactive thiostrepton totally abolished this binding (data not given). Accordingly we conclude that this preparation was radiochemically pure. Ideally, one might have hoped that all the purified drug would bind to ribosomes and we presume that the nature of the assay system employed accounts for the discrepancy. As ribosomes progressed down the gel-column and the concentration of free drug diminished, some of the bound material evidently dissociated from the ribosomes to maintain the binding equilibrium.

Binding of purified [^{35}S]thiostrepton to ribosomes of S. azureus and S. coelicolor. Having ascertained that ribosomes of *S. azureus* were resistant to inhibition by thiostrepton, we sought to determine whether this resistance originated from an inability of the ribosomes to bind the drug or from a lack of inhibitory response to bound drug. As shown in Fig. 2, ribosomes from *S. azureus* bound [^{35}S]thiostrepton only poorly in comparison with those from *S. coelicolor* or from *E. coli*. At present we cannot compare the binding constants with ribosomes from all these sources since the binding to *S. azureus* ribosomes is so poor.

DISCUSSION

The organisms which produce thiostrepton and micrococcin are each resistant to their own toxic product; however, the mechanisms of resistance are not similar. The micrococcin producer (Su's *Micrococcus*) possesses ribosomes which are susceptible to micrococcin and we therefore presume either that this organism possesses an enzyme capable of detoxifying micrococcin or (more likely, perhaps) that resistance is derived from the permeability properties of the cell membrane. Similar considerations also apply in the case of *B. pumilis* (the micrococcin P producer) although we have not presented the data here. In contrast, *S. azureus* (the thiostrepton producer) possesses ribosomes which are totally refractory to thiostrepton, apparently by virtue of a greatly diminished ability to bind the drug when compared with ribosomes from *E. coli* or from *S. coelicolor*, which is thiostrepton susceptible. Moreover, in agreement with ideas expressed elsewhere (E. Cundliffe and P. Dixon, Antimicrob. Agents Chemother., in press) concerning the likely identity of the ribosomal binding sites of members of the thiostrepton group, we have shown here that cross resistance to this group of compounds can be exerted at the ribosomal level.

One point which has intrigued us concerns the possibility that *Streptomyces* ribosomes might be modified in the direction of drug resistance to coincide with the onset of drug production. If so, and if such modification involved a simple, specific substitution to blockade the binding site of the drug, exploitation of this phenomenon might afford a potent means of locating antitiotic binding sites on ribosomes. Obviously, to test this hypothesis it would be necessary to produce ribosomes from streptomycetes in different phases of growth and one is immediately faced with the problem that many of these organisms grow in spherical clumps which probably contain mycelia in different growth phases. Thus, although our experiments were carried out with ribosomes derived from a "young" culture of *S. azureus*, we do not consider this point to be unequivocally settled at this time, although the results presented here would appear to negate the above hypothesis.

Whether the phenomenon of intrinsic resistance of a streptomycete to its own toxic product is part of a general pattern remains to be determined. In particular, it will be of interest to study the organisms which produce aminoglycoside antibiotics and which may have available an enzymic mechanism of resistance (1). Whether such organisms would need to possess drug-resistant ribosomes remains to be seen.

While this work was in its final stages of completion we learned (9) that *Streptomyces erythreus* (an erythromycin producer) possesses ribosomes which are not inhibited by erythromycin nor do they bind the drug; results analogous to those which we report here.

ACKNOWLEDGMENTS

We thank the Wellcome Trust for a senior research fellowship (to E.C.) and for research funds, and the Medical

Research Council for a research studentship (to P.D.D.) and for a research grant (to E.C.).

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