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Inhibition of Ribosomal A Site Functions by Sporangiomycin and Micrococcin

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Sporangiomycin and micrococcin inhibit the binding of aminoacyl-transfer ribonucleic acid into the ribosomal A site in intact bacterial protoplasts. They also prevent the assembly of [ribosome-elongation factor G-guanine nucleotide] complexes in vitro and compete with [^{35}S]thiostrepton for ribosomal binding sites. We conclude that micrococcin and sporangiomycin block the ribosomal A site in the vicinity of the complex guanosine triphosphatase center and so resemble thiostrepton in their modes of action.

The peptide antibiotics thiostrepton, siomycin, and thiopeptin possess closely related (possibly identical) modes of action upon protein synthesis. They bind to the bacterial 50S ribosomal subunit and inhibit, in various in vitro assays, the hydrolysis of guanosine triphosphate (GTP) catalyzed by ribosomes in the presence of elongation factor G (EF G), the formation of [ribosome-EF G-guanine nucleotide] complexes and their stabilization by fusidic acid, the binding of aminoacyl-transfer ribonucleic acid (tRNA) into the ribosomal A site dependent upon elongation factor Tu (EF Tu), and the associated hydrolysis of GTP (for a review see reference 6). However, in intact bacterial protoplasts, thiostrepton specifically inhibits the binding of aminoacyl-tRNA to ribosomes (5) and does not affect the translocation reaction even though this latter process involves, in part, the EF G-dependent guanosine triphosphatase (GTPase) reaction. Subsequently, similar selectivity was reported when thiostrepton (3) or siomycin (4) was added to a cell-free system actively synthesizing protein. These various results were rationalized and unified by the suggestion (5, 10, 12) that EF G and EF Tu may bind to ribosomes in a mutually exclusive fashion and that thiostrepton (or siomycin or thiopeptin) might, under particular conditions, bind to a single ribosomal site so as to prevent binding of either factor. According to our version of this scheme (the single ribosomal GTPase model), thiostrepton can bind to active ribosomes only in the post-translocation state when the A site is empty, and we locate the GTPase center together with the binding sites for EF G, EF Tu, and the drug in the A site on the 50S ribosomal subunit (5). This model is supported by numerous observations (2, 7, 11, 17, 18) and is assumed to apply to both pro- and eukaryotic ribosomes.

Here we show that two other antibiotics, sporangiomycin and micrococcin, resemble thiostrepton, siomycin, and thiopeptin in various assay systems and, moreover, that there is competition for ribosomal binding sites between radioactive thiostrepton and each of these other compounds.

MATERIALS AND METHODS

Experiments in intact cells. *Bacillus megaterium* strain KM was grown for several generations with [^{32}P]phosphate to label ribosomes to a steady state and was then converted to protoplasts using lysozyme (8). Protoplasts were incubated at 37 C for 30 s with [^{3}H]leucine (50 Ci/mmol) to label nascent peptides before antibiotics were added (100 μg/ml final concentration) as in Table 1. Incubation was continued at 37 C for 2 min and then puromycin (25 μg/ml final concentration) was added for a further 60 s. Samples of protoplasts were lysed, degraded by mild treatment with ribonuclease, and analyzed on sucrose density gradients as described elsewhere (5, 8). Nascent peptides on ribosomes were estimated from the specific radioactivity (^{3}H in 70S monosomes/^{32}P in total ribosomes). Polyribosomes were estimated as (^{32}P in 70S monosomes/^{32}P in total ribosomes).

Ribosomes. These were prepared from *Escherichia coli* B or *B. megaterium* KM which were lysed using the French pressure cell in a buffer containing: tris(hydroxymethyl)aminomethane-chloride (pH 7.6), 10 mM; magnesium acetate, 10 mM; NH_4Cl, 50 mM; and dithiothreitol, 1 mM. Ribosomes were washed seven times by centrifugation through a similar buffer containing 1.0 M NH_4Cl and were finally resuspended, for use, in the original buffer containing 50 mM NH_4Cl.

Formation of [ribosome-EF G-guanine nucleotide] complexes. The buffer used for these experiments (Table 2) contained: tris(hydroxymethyl)aminomethane-chloride (10 mM), pH 7.5; magnesium ace-

TABLE 1. *Effects of antibiotics on the puromycin reaction and upon the state of aggregation of polyribosomes in vivo*[a]

Additions	Nascent peptides on ribosomes after puromycin treatment (% of those present when puromycin added)	Ribosomes involved in polysomes prior to addition of puromycin (% of cellular total)
ME_2SO (control)		74
Thiostrepton then puromycin	10	69
Sporangiomyin then puromycin	21	72
Micrococcin then puromycin	26	66
Thermothiocin then puromycin	60	68

[a] With the exception of puromycin, antibiotics used here were dissolved in dimethyl sulfoxide (ME_2SO) which was present in incubations at a final concentration of 1% (vol/vol). This amount of ME_2SO was added to controls and was found (data not given) to be without inhibitory effect.

tate, 20 mM; NH_4Cl, 10 mM; and dithiothreitol, 1 mM. Incubation 1 was as follows: *E. coli* ribosomes (10 pmol) were incubated with or without drug as indicated (Table 2) for 15 min at 0 C in a total volume of 20 μl. Incubation 2 (5 min at 0 C) was the following components added as a mixture in a 50-μl volume: EF G, 50 pmol; [ring-^{3}H]GTP (13.3 Ci/mmol), 49 pmol; and fusidic acid, 2.5 mM (final concentration). Incubation 2 was terminated by the addition of 3 ml of ice-cold buffer containing 0.2 mM fusidic acid. Ribosomes were collected on membrane filters (Millipore HAWP filters) (0.45-μm pore size) which were washed four times with cold buffer containing 0.2 mM fusidic acid. 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 used to correct experimental values which were themselves the means of duplicates.

Preparation of [^{35}S]thiostrepton. Radioactive thiostrepton was prepared by growth of *Streptomyces azureus* ATCC 14921 with [^{35}S]sulfate followed by centrifugation and extraction of the mycelial pellet with chloroform. After concentration of this extract, *E. coli* ribosomes were added to bind all the drug present and the ribosomes were then recovered by chromatography on Sepharose 6B. Thiostrepton was removed from these ribosomes by prolonged shaking with chloroform and the extract was dried down and taken up in dimethyl sulfoxide. The resultant solution contained radiochemically pure [^{35}S]thiostrepton (120 pmol/5 μl) at a specific radioactivity of 25 mCi/mmol. This method was as described by Dixon et al. (8a).

Binding of radioactive thiostrepton to ribosomes. Buffer used here contained tris(hydroxymethyl)aminomethane-chloride (10 mM), pH 7.6, magnesium acetate (10 mM), NH_4Cl (50 mM), and dithiothreitol (1 mM). Ribosomes from *B. megaterium* (34 pmol in 40 μl of buffer) were incubated with [^{35}S]thiostrepton (5 μl in dimethyl sulfoxide, equivalent to 120 pmol at 50 counts/min per pmol) for 15 min at room temperature. 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. When the effects of other drugs on the binding of [^{35}S]thiostrepton were to be investigated (Table 3), ribosomes were incubated with such drugs (added as 5 μl in buffer or dimethyl sulfoxide) for 15 min at room temperature prior to the addition of [^{35}S]thiostrepton as above. In controls, prior exposure of ribosomes to dimethyl sulfoxide at a concentration of 11% (vol/vol) did not affect the subsequent binding of [^{35}S]thiostrepton to those ribosomes.

Materials. Radiochemicals were obtained from the Radiochemical Centre, Amersham, England. The sources of drugs were as follows: thiostrepton, The Squibb Institute for Medical Research, Princeton, N.J.; sporangiomycin and thermothiocin, Gruppo Lepetit, Milano; siomycin, K. Tanaka, Shionogi & Co. Ltd., Osaka; thiopeptin, Fujisawa Pharm. Co. Ltd., Osaka; althiomycin and sparsomycin, The Upjohn Co., Kalamazoo; Micrococcin was a kind gift from N. Heatley, University of Oxford; and micrococcin P was provided by J. Walker, National Institute for Medical Research, Mill Hill, London, England. These two compounds are believed to be identical and they responded indistinguishably in all assays described here. The following molecular weights were

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

Drug added	Amount (pmol)	pmol of GTP complexed (% of control)
Thiostrepton	155	21
Sporangiomycin	140	0
Micrococcin	208	13
Thermothiocin	167	100
Althiomycin	353	100
Chlortetracycline	433	100

[a] Ribosomes from *E. coli* were used here. In controls, 10 pmol of ribosomes complexed approximately 6 pmol of guanine nucleotide (presumably as guanine dinucleotide) and this was not affected by concentrations of ME_2SO of up to 30% (vol/vol). With the exception of chlortetracycline, antibiotics used here were dissolved in ME_2SO which was present in incubation 1 at a final concentration of 25% (vol/vol).

TABLE 3. *Effects of antibiotics upon the binding of [^{35}S]thiostrepton to B. megaterium ribosomes*

Drug added	Amount (nmol)	Inhibition of binding of [^{35}S]thiostrepton (%)
Sporangiomycin ...	14	>95
Micrococcin	21	31
Thiopeptin	13	>95
Siomycin	12	>95
Thiostrepton	16	>95
Chloramphenicol ..	32	8
Sparsomycin	28	13
Althiomycin	28	14
Thermothiocin	17	20
Fusidic acid	36	7

assumed: thiostrepton (1,616), siomycin (1,712), sporangiomycin (1,800), micrococcin/micrococcin P (1,208; J. Walker, personal communication, a revision of the published value), thiopeptin (1,942), althiomycin (708), and thermothiocin (1,500).

RESULTS

Effects of sporangiomycin and micrococcin in intact cells. When added to intact bacterial protoplasts at concentrations well in excess of those required to stop protein synthesis, micrococcin and sporangiomycin preserved cellular polyribosomes virtually intact, but did not prevent the subsequent release by puromycin of 75 to 80% of the nascent peptide chains from those polyribosomes (Table 1). Thiostrepton behaved similarly and, as we have previously argued (5), this pattern of behavior indicates inhibition of the binding of aminoacyl-tRNA into the ribosomal A site.

Formation of [ribosome-EF G-guanine nucleotide] complexes and the effects of antibiotics. To investigate more precisely the mechanism whereby micrococcin and sporangiomycin block the A site, their effects were compared with those of chlortetracycline (which also blocks the A site in vivo; 8) in an assay involving the formation and stabilization of [ribosome-EF G-guanine nucleotide] complexes in the presence of fusidic acid. The results are given in Table 2 together with the effects of althiomycin and thermothiocin. Again, thiostrepton, micrococcin, and sporangiomycin behaved similarly in inhibiting complex formation and differed in this respect from the other drugs tested.

Effects of antibiotics on the binding of [^{35}S]thiostrepton to ribosomes. The effects of a number of antibiotics (all of them inhibitors of the 50*S* ribosomal subunit; see reference 6) on the binding of [^{35}S]thiostrepton to ribosomes of *B. megaterium* are given in Table 3. Binding was totally prevented by prior exposure of ribosomes to sporangiomycin, siomycin, or thiopeptin and was also depressed by micrococcin, albeit to a much lesser extent. Other antibiotics tested gave very little inhibition.

DISCUSSION

We have established, in a system actively synthesizing protein, the modes of action of sporangiomycin and micrococcin and have differentiated these drugs in their action from thermothiocin and althiomycin (other sulfur-containing polypeptides). The latter extensively inhibit the puromycin reaction in vivo (Table 1; 1) and are probably inhibitors of peptidyl transferase reactions in agreement with the previously observed action of althiomycin in vitro (14). In contrast, sporangiomycin and micrococcin block the ribosomal A site in the vicinity of the GTPase site and the associated binding site of EF G (Table 2). Not all drugs which block the ribosomal A site in vivo inhibit the formation of [ribosome-EF G-guanine nucleotide] complexes in vitro. Thus, chlortetracycline (8) did not inhibit complex formation (Table 2) nor did thermothiocin or althiomycin. While these results are at variance with a previous report (15) that micrococcin does not inhibit the formation of such complexes, they are entirely compatible with earlier observations concerning the action of sporangiomycin. Thus (19), the hydrolysis of GTP by ribosomes and EF G was inhibited by sporangiomycin (the formation of complexes of the type studied here was not examined by these authors) and, in other hands (16), the drug inhibited the EF Tu-dependent binding of aminoacyl-tRNA to ribosomes in vitro. Our demonstration (Table 1) that effects of micrococcin and sporangiomycin upon this latter reaction predominate in vivo over other effects which the drugs may exert in vitro again emphasizes the similarities between the modes of action of these two compounds and that of thiostrepton.

As expected, those drugs which closely resembled thiostrepton in their modes of action competed with [^{35}S]thiostrepton for ribosomal binding sites, although micrococcin did so relatively poorly. Although reciprocal experiments could not be carried out due to a lack of other radioactive antibiotics, it is tempting to conclude that the ribosomal binding sites for thiostrepton, siomycin, thiopeptin, sporangiomycin, and probably also micrococcin are either identical or are intimately associated in the 50*S* moiety of the A site. An earlier observation (9) that a

strain of *Bacillus subtilis* possessing thiostrepton-resistant ribosomes was also resistant to micrococcin may well indicate that the two drugs bind to related ribosomal sites even though the ribosomes of that strain were not shown to be micrococcin resistant. Conversely, the susceptibility towards thiostrepton of another strain which possessed micrococcin-resistant ribosomes indicates that these drugs bind to overlapping, rather than identical, sites. Since chloramphenicol and sparsomycin are thought to bind to (separate) sites in the peptidyl transferase A site recognition region (20) but do not affect the binding of thiostrepton to the GTPase center of the A site (Table 3), the complexity of the A site (already obvious) is emphasized.

Results similar to ours with micrococcin have recently been reported elsewhere (13).

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