The Mode of Action of Nosiheptide (Multhiomycin) and the Mechanism of Resistance in the Producing Organism

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The mode of action of nosiheptide (multhiomycin) on bacterial protein synthesis is closely similar to that of thiostrepton. Both antibiotics inhibit functions of elongation factors Tu and G and greatly reduce the synthesis of guanosine penta- and tetraphosphates in response to stringent factor. Furthermore, the actinomycetes which produce these antibiotics defend themselves against their products in similar fashion. This involves specific pentose-methylation of 23S ribosomal RNA.

INTRODUCTION

A number of sulphur-containing polypeptide antibiotics selectively inhibit protein synthesis in bacterial extracts, although some of them fail to penetrate Gram-negative cells. Among such agents are thiostrepton, siomycin, sporangiomycin and thiopeptin (the 'thiostrepton group' of antibiotics) which are closely related in structure and possess apparently identical modes of action (for a review, see Cundliffe, 1980). In contrast, other sulphur-containing peptides such as althiomycin, thermothiocin and actinotiocin which have, on occasion, been compared with thiostrepton (Tamura *et al.*, 1973) are now known to act differently (Pestka & Brot, 1971; Cundliffe & Dixon, 1975).

Multhiomycin, produced by *Streptomyces antibioticus* (Tanaka *et al.*, 1970*a*), has recently been shown to be identical to nosiheptide, the product of *Streptomyces actuosus*, and to be related in structure to thiostrepton – not least to the extent that both molecules contain five thiazole rings (Prange *et al.*, 1977). To date, relatively little has been reported concerning the action of this compound; however, multhiomycin selectively inhibited protein synthesis in intact cells of *Bacillus subtilis* and in extracts of *Escherichia coli* (Tanaka *et al.*, 1970*b*). It also seemed clear from these studies that multhiomycin probably acted by binding directly to ribosomes. Closer examination revealed that the drug inhibited partially the enzymic binding of aminoacyl-tRNA to the [ribosome·mRNA] complex together with the associated hydrolysis of GTP, but only when present at quite high molar excess over ribosomes (Tanaka *et al.*, 1971). Since thiostrepton group antibiotics also act in this way, in addition to affecting other partial reactions of protein synthesis, we have examined further the mechanism of action of nosiheptide (multhiomycin) and compared its effects with those of thiostrepton. Actinotiocin and thermothiocin, about whose modes of action little is known, were also included in various assays.

Actinomycetes which produce thiostrepton, siomycin or sporangiomycin defend themselves against their products via similar patterns of post-transcriptional modification of ribosomal RNA (rRNA). Thus, methylation at a single site within 23S rRNA leads to ribosomes which are refractory to the action of these drugs and to which [³⁵S]thiostrepton does not bind detectably (Cundliffe, 1978; Thompson & Cundliffe, 1980). The methylated residue implicated in this mode of self-defence is 2'-O-methyladenosine (Cundliffe & Thompson, 1979). However, such ribosomes remain sensitive to the action of actinotiocin, thermothiocin

and other antibiotics not belonging to the thiostrepton group (E. Cundliffe & J. Thompson, unpublished data). Accordingly, we wished to determine whether pentose-methylated ribosomes are also resistant to nosiheptide and whether *S. actuosus* utilizes a similar mechanism of resistance to its product.

METHODS

Preparation of *E. coli* ribosomes and sub-ribosomal core particles. Methods for the preparation of salt-washed ribosomes and post-ribosomal supernatant (S100) and their utilization in polyphenylalanine synthesis in response to polyuridylic acid [poly(U)] have been described in full elsewhere (Cundliffe *et al.*, 1979). The preparation of core particles and 'split proteins' by exposure of ribosomes to LiCl (here we used 3 M-LiCl) was as described, except that split proteins were concentrated and stored in 'reconstitution buffer' [containing 20 mM-Tris/HCl (pH 7.6 at 20 °C), 20 mM-MgCl₂, 350 mM-NH₄Cl, 3 mM-2-mercaptoethanol].

Methylation of core particles and re-addition of split proteins. The 3 M-LiCl core particles from *E. coli* ribosomes (126 pmol) were incubated at 35 °C with *S. actuosus* S100 (60 µl; arbitrary amount) in 210 µl buffer [containing 50 mM-*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES)/KOH (pH 7.5 at 20 °C), 10 mM-MgCl₂, 50 mM-NH₄Cl, 3 mM-2-mercaptoethanol] in the presence and absence of *S*-adenosylmethionine (SAM; 0.5 mM). At intervals, samples (50 µl) containing 30 pmol cores were mixed with 30 µl (96 pmol equivalents) 3 M-LiCl split proteins and incubation was continued at 20 °C for 10 min. One pmol equivalent of split proteins is defined as those proteins released from one pmol 70S ribosomes during their conversion to core particles.

Binding of [³⁵S]thiostrepton to reconstituted ribosomal particles. This was carried out essentially as described elsewhere (Thompson *et al.*, 1979). Radiolabelled drug (39 pmol; 480 c.p.m. pmol⁻¹) was added directly to reconstitution mixtures containing 30 pmol 3 M-LiCl core particles and 96 pmol 3 M-LiCl split proteins, as above. Incubation was continued at 20 °C for 10 min before 10 µl of 5% (w/v) suspension of activated charcoal ('Norit') was added to adsorb unbound drug. After 5 min at 20 °C, charcoal was removed by centrifugation at 12 000 g for 2 min and ribosome-bound thiostrepton in the supernatant was estimated by liquid-scintillation spectrometry.

Uncoupled hydrolysis of GTP by ribosomes and elongation factor EF-G. Reaction mixtures (75 µl) contained 2.4 pmol 70S ribosomes from *E. coli* together with 50 pmol factor EF-G and 9 nmol [γ -³²P]GTP [62.3 µCi µmol⁻¹ (2.305 MBq µmol⁻¹), i.e. approximately 135 c.p.m. pmol⁻¹ in buffer [containing 15 mM-Tris/HCl (pH 7.6 at 20 °C), 12 mM-MgCl₂, 100 mM-NH₄Cl, 4 mM-2-mercaptoethanol]. During incubation at 30 °C, samples (20 µl) were removed at intervals into 20 µl ice-cold 1 M-perchloric acid to terminate hydrolysis. Unhydrolysed, radioactive GTP was removed by addition of 200 µl of 5% (w/v) suspension of activated charcoal ('Norit') in water followed by centrifugation at 12 000 g for 2 min to remove both the charcoal and the macromolecular precipitate. Radioactivity ([³²P]phosphate) in the supernatant was estimated by liquid-scintillation spectrometry. Purified factor EF-G from *E. coli* was a kind gift from Professor James Bodley, Department of Biochemistry, University of Minnesota, Minneapolis, U.S.A.

Synthesis of guanosine penta- and tetraphosphates *in vitro*. This was carried out using 70S ribosomes, unfractionated tRNA and crude stringent factor (all from *E. coli*) together with poly(U), GTP and [γ -³²P]ATP (80 c.p.m. pmol⁻¹) as described elsewhere (Stark & Cundliffe, 1979*a*). Crude stringent factor was prepared by ammonium sulphate fractionation of a 1 M-NH₄Cl ribosomal wash according to Block & Haseltine (1975). Reaction mixtures were incubated at 37 °C for 40 min before nucleotide products were separated by chromatography on poly(ethyleneimine)-cellulose plates and located by autoradiography overnight. Radioactivity incorporated into guanosine penta- and tetraphosphates was determined by cutting out appropriate pieces of the chromatogram followed by liquid-scintillation spectrometry (for details see Stark & Cundliffe, 1979*a*).

Cell-free protein synthesis in extracts of actinomycetes. The growth and extraction of actinomycetes and the preparation of salt-washed ribosomes and S100 suitable for the study of polyphenylalanine synthesis *in vitro* have recently been described (Thompson & Cundliffe, 1980).

Preparation and characterization of [³⁵S]nosiheptide. Spores of *Streptomyces actuosus* NRRL 2954 were used to inoculate 50 ml of medium containing (per litre) 10 g peptone, 15 g malt extract, 20 g glucose, 3 g yeast extract and 20 g maltose. After 2 d at 30 °C, 3.5 mCi (130 MBq) carrier-free [³⁵S]sulphate was added followed 1 d later by a similar addition. Incubation was continued at 30 °C with gentle shaking until, after 8 d in the presence of [³⁵S]sulphate, the whole culture was extracted repeatedly by shaking with chloroform. The extract was taken down to a small volume by rotary flash evaporation at 37 °C and passed twice over a Whatman DE 52 column (5 × 1 cm) in chloroform. This removed brown material together with about one-third of the radioactivity. After drying the eluate, crude [³⁵S]nosiheptide was taken up in dimethyl sulphoxide leaving about 25% of the radioactivity in the form of a waxy residue. Of the radiolabelled material in this preparation, about 70% bound to *E. coli* ribosomes and, of this, two-thirds was evidently [³⁵S]nosiheptide: its binding to *E. coli* ribosomes was competitive with that of authentic nosiheptide and it did not bind detectably to ribosomes of *S. actuosus*. In

contrast, the remaining one-third of the ribosome-binding material attached equally well to ribosomes from both organisms and such 'non-specific' binding was not prevented by prior addition of nosiheptide. The specific radioactivity of [^{35}S]nosiheptide was established in the usual way by dilution with authentic drug (which was assumed to be 100% pure) followed by ribosome-binding studies. The initial specific radioactivity was close to 400 c.p.m. pmol $^{-1}$, the apparent stoichiometry of binding to *E. coli* ribosomes was close to 1:1 and the total yield of [^{35}S]nosiheptide was approximately 300 nmol (about 370 μg). In all these experiments, binding of drug to ribosomes was determined following the removal of unbound material by adsorption to activated charcoal as above (see also Thompson *et al.*, 1979).

Binding of [^{35}S]nosiheptide to RNA-protein complexes. Complexes were formed between purified *E. coli* 23S rRNA (52 pmol), or total RNA from 70S ribosomes (52 pmol), and ribosomal protein L11 (186 pmol) by incubating them together for 10 min at 20 °C as described elsewhere (Thompson *et al.*, 1979) except that here the buffer contained 10 mM-MgCl $_2$. Then, [^{35}S]nosiheptide (approximately 120 pmol in 5 μl dimethyl sulphoxide) was added for a further 10 min at 20 °C. The final incubation volume was 100 μl . Subsequently, unbound drug was removed by adsorption to activated charcoal followed by centrifugation (see Thompson *et al.*, 1979). Complexes of rRNA with protein L11 evidently bound both nosiheptide and some other unidentified material present in the radiolabelled drug preparation. Specific binding of [^{35}S]nosiheptide was dependent upon the presence of both 23S rRNA and protein L11 and was prevented by prior addition of authentic drug. Non-specific binding occurred to similar extents with RNA-L11 complexes, with rRNA alone (but not with protein L11 alone) and with 70S ribosomes (see above). This background was subtracted from data presented in Table 2 and amounted to about one-third of the total when protein L11 was present.

Ribosomal protein L11 from *E. coli* was kindly provided by Dr Jan Dijk and was prepared according to Dijk & Littlechild (1979).

RESULTS

Nosiheptide is a potent inhibitor of protein synthesis in extracts of *E. coli* (Tanaka *et al.*, 1970*b*). In our hands, following incubation with an equimolar amount of the drug, *E. coli* ribosomes were almost totally inhibited in polyphenylalanine synthesis in vitro (data not given). Ribosomes from *Streptomyces coelicolor* were equally sensitive to the drug (see below). In a further series of experiments, we confirmed earlier observations (Tanaka *et al.*, 1971) that nosiheptide inhibits, albeit incompletely, the enzymic binding of phenylalanyl-tRNA to the [ribosome·poly(U)] complex. Our results are merely stated here (see Stark & Cundliffe, 1979*b*, for details of methods). Thus, when present at 100-fold molar excess over ribosomes, nosiheptide inhibited the enzymic binding reaction by about 30%, in contrast to thiostrepton which inhibited binding by about 70% under similar conditions. We did not examine the hydrolysis of GTP which normally accompanies such binding; however, partial inhibition of this reaction by nosiheptide has also been reported (Tanaka *et al.*, 1971).

Continuing our comparison of the effects of nosiheptide and thiostrepton, we examined ribosomal functions dependent upon factor EF-G. This protein functions in the complex translocation reaction of polypeptide chain elongation during which one molecule of GTP is hydrolysed per active ribosome. Neither factor EF-G nor the ribosome alone possesses GTPase activity but together, in vitro, they catalyse multiple rounds of GTP hydrolysis in a manner uncoupled from peptide chain elongation. Only antibiotics of the thiostrepton group have been shown to inhibit such uncoupled hydrolysis of GTP, hence we were particularly interested to examine nosiheptide in this system. As shown in Fig. 1, nosiheptide was, at best, a poor inhibitor of GTP hydrolysis. However, given that other inhibitors of protein synthesis, including actinotycin and thermothiocin, routinely fail to inhibit this system to any detectable extent (Pestka & Brot, 1971; also Fig. 1), the levels of inhibition (around 20%) observed with nosiheptide appear significant.

A related assay, also inhibited exclusively by thiostrepton group antibiotics, involves the formation of [ribosome·EF-G·guanine nucleotide] complexes and their stabilization in the presence of fusidic acid (Bodley *et al.*, 1970; Weisblum & Demohn, 1970). Such complexes can, in vitro, be formed directly either from GDP or from GTP in which case the latter is normally hydrolysed. Nosiheptide proved to be a potent inhibitor of the formation and/or stabilization of [ribosome·EF-G·guanine nucleotide] complexes, although not as effective as

Fig. 1

Fig. 1. Uncoupled hydrolysis of GTP by ribosomes and factor EF-G in the presence of drugs. Ribosomes were incubated with drugs for 5 min at 30 °C prior to addition of factor EF-G and [γ - 32 P]GTP. Hydrolysis of GTP was then followed over a 10 min time course as described in Methods. Data were computed for the end-points of such incubations, by which time, in controls, approximately 1350 pmol GTP had been hydrolysed per pmol ribosomes. Controls contained dimethyl sulphoxide (usually 1%, v/v, final concentration) in which drug solutions were prepared. Drugs: ●, thioestrepton; ○, nosiheptide; ■, thermothiocin; □, actinotiocin.

Fig. 2

Fig. 2. Synthesis of guanosine penta- and tetraphosphates in response to stringent factor in the presence of drugs. Ribosomes were incubated with drugs (dimethyl sulphoxide was added to controls) for 10 min at 20 °C prior to addition of other assay components (see Methods). In controls, approximately 12% of the radioactivity added as [γ - 32 P]ATP was incorporated into guanosine polyphosphates. Drugs: ●, thioestrepton; ○, nosiheptide; ■, thermothiocin; □, actinotiocin; Δ, bottromycin.

Table 1. *Effects of antibiotics on the formation of [ribosome·EF-G·guanine nucleotide] complexes in the presence of fusidic acid*

Reaction mixtures contained 20 pmol *E. coli* ribosomes, 50 pmol factor EF-G, 100 pmol [*ring*- 3 H]GTP (13.3 Ci pmol $^{-1}$) and 3 mM-sodium fusidate; for details, see Stark & Cundliffe (1979*b*). Drugs were added in solution in dimethyl sulphoxide which was also included in controls.

Drug added	Molar excess of drug over ribosomes	[Ribosome·EF-G·guanine nucleotide] complexes formed	
		pmol per 100 pmol ribosomes	% of control
None	—	63.8	100
Nosiheptide	3.7	12.4	19.4
	37	11.9	18.7
	125	12.4	19.4
	800	10.5	16.5
Thioestrepton	3.7	3.6	5.6
	37	3.5	5.5
	125	2.8	4.4
	600	3.1	4.9

Table 2. *Binding of [³⁵S]nosiheptide to RNA-protein complexes*

Complexes were formed by incubating purified *E. coli* 23S rRNA (52 pmol) or total RNA from 70S ribosomes (RNA 70, 52 pmol) with *E. coli* ribosomal protein L11 (186 pmol). [³⁵S]Nosiheptide (120 pmol) was then added and its binding to the complexes was assayed. For details, see Methods.

rRNA input	Protein L11	[³⁵ S]Nosiheptide bound (pmol)
23S rRNA	+	56
RNA 70	+	59
RNA 70	—	0
RNA 70	+	2*

* Thiostrepton (75 pmol) added prior to nosiheptide.

thiostrepton (Table 1). Again, this indicates similarities in the action of the two drugs, particularly since the assay is unaffected by a large number of other antibiotics which inhibit protein synthesis (Pestka & Brot, 1971; E. Cundliffe & J. Thompson, unpublished observations) including actinotycin and thermothiocin.

One further ribosomal reaction was examined and was found to be extremely sensitive to inhibition by nosiheptide. This involved the synthesis in vitro of the regulatory nucleotides guanosine penta- and tetraphosphates (the so-called 'magic spot' compounds) in response to stringent factor, mRNA and cognate deacylated tRNA. Pyrophosphate transfer from ATP to the 3'-hydroxyl of GTP or GDP occurs under such conditions in a reaction which appears to involve the ribosomal A site. Both thiostrepton and nosiheptide inhibited this reaction potently whereas other peptides such as actinotycin, thermothiocin and bottromycin were virtually without effect (Fig. 2). Production of guanosine polyphosphates is inhibited by drugs such as chlortetracycline which prevent the binding of mRNA-cognate tRNA into the ribosomal A site (Pedersen *et al.*, 1973). Hence, it is not clear from our data whether nosiheptide and thiostrepton act in that manner or whether they interfere directly with the binding or action of stringent factor.

From these results, we concluded that nosiheptide resembled thiostrepton in various aspects of its action but that, generally, thiostrepton acted more potently. In accord with this, nosiheptide proved to be a weak inhibitor of the binding of thiostrepton to *E. coli* ribosomes. However, when present at 1000-fold molar excess over [³⁵S]thiostrepton, nosiheptide inhibited binding of the latter by 85–90% (data not given). The reciprocal experiments were more revealing. [³⁵S]Nosiheptide bound firmly to *E. coli* ribosomes (but not to those of *S. actuosus*) and such binding was powerfully inhibited by thiostrepton (data not given). Further, [³⁵S]nosiheptide bound stoichiometrically to the complex of 23S rRNA with ribosomal protein L11 (Table 2), and thiostrepton, which also binds to such complexes, was again inhibitory.

These data were supported by the results of experiments with thiostrepton-resistant mutants of *Bacillus megaterium*, which lack a protein (designated BM-L11) normally present in the 50S ribosomal subunit (Cundliffe *et al.*, 1979). Protein BM-L11 is the immunological and functional homologue of ribosomal protein L11 of *E. coli*. Since thiostrepton normally binds to the complex of 23S RNA with protein (BM) L11, ribosomes from the mutants bind the drug poorly in comparison with those from the wild-type. As expected from the data presented in Table 2, the thiostrepton-resistant mutants were cross-resistant to nosiheptide (data not given). Interestingly, however, they remained sensitive to actinotycin and thermothiocin.

Since thiostrepton and nosiheptide evidently bound to related (or even identical) sites on the *E. coli* ribosome (albeit with differing affinity), we examined the ribosomes from the organisms which produce the two antibiotics. Initially, we observed that *Streptomyces azureus* (which produces thiostrepton) and *Streptomyces actuosus* (the nosiheptide producer) were unaffected by thiostrepton or nosiheptide when challenged on growth plates with

Fig. 3. Synthesis of polyphenylalanine by *Streptomyces* ribosomes in the presence and absence of drugs. Ribosomes from *S. coelicolor* (a) or *S. actuosus* (b) were incubated with drugs (dimethyl sulphoxide was added to controls) for 10 min at 30 °C prior to the addition of other assay components. In each case post-ribosomal supernatant from *S. coelicolor* was employed. Samples contained 6 or 7 pmol ribosomes; [^{14}C]phenylalanine was used at a specific radioactivity of 513 mCi mmol $^{-1}$ (about 1000 c.p.m. pmol $^{-1}$). (a) ○, control; ●, plus thioestrepton (100 $\mu\text{g ml}^{-1}$) or nisin (100 $\mu\text{g ml}^{-1}$) – effects indistinguishable; (b) ○, control or plus thioestrepton (100 $\mu\text{g ml}^{-1}$); ●, plus nisin (100 $\mu\text{g ml}^{-1}$).

Table 3. *Effects of methylation of E. coli ribosomal cores by S. actuosus extract*

Mixtures containing 3 M-LiCl core particles from *E. coli* ribosomes and *S. actuosus* S100 were incubated with or without SAM for the periods indicated. Samples were then mixed with 3 M-LiCl 'split proteins', and binding of [^{35}S]thioestrepton to the reconstituted particles was assayed. For details, see Methods. In the absence of split proteins, binding of [^{35}S]thioestrepton to cores was 0.02 pmol per pmol.

Time of incubation (min)	Cofactor (SAM)	[^{35}S]Thioestrepton bound (pmol per pmol reconstituted particles)
0	—	1.00
20	+	0.72
20	—	0.89
45	+	0.57
45	—	0.88
120	+	0.09
120	—	0.88

antibiotic assay discs (data not given). Subsequently, ribosomes from *S. actuosus* proved to be totally insensitive to either drug when synthesizing polyphenylalanine in vitro, in marked contrast to those from *Streptomyces coelicolor* (Fig. 3). In this respect, ribosomes from *S. actuosus* were indistinguishable from those of *S. azureus* (data not given).

More detailed examination of *S. actuosus* revealed that rRNA from this organism was not a substrate for the 'thioestrepton-resistance methylase' of *S. azureus* (data not given). This enzyme, which has been extensively purified (Thompson & Cundliffe, 1981), is active on 23S rRNA from bacteria of a range of genera including *Streptomyces*, and its failure to act on rRNA from *S. actuosus* suggested that the latter organism might contain a similar methylase. This hypothesis was substantiated in a series of experiments in which post-ribosomal supernatant (S100) of *S. actuosus* was employed as a source of methylase activity. The

crucial experiment is summarized in Table 3. Ribosomal core particles from *E. coli* were incubated with *S. actuosus* S100 in the presence and absence of *S*-adenosylmethionine (SAM) as cofactor. Then, 'split proteins' were added back to the cores and the ability of the reconstituted ribosomes to bind [³⁵S]thiostrepton was assayed. In this way, the presence in *S. actuosus* of a methylase capable of rendering ribosomes refractory to thiostrepton was revealed. In other experiments, employing [*methyl*-³H]SAM as cofactor and rRNA from *S. coelicolor* as substrate, we determined that crude methylase from *S. actuosus* introduced methyl groups exclusively into 23S rRNA with stoichiometry close to unity (data not given). We then subjected such RNA to enzymic digestion and analysis as described elsewhere (Cundliffe & Thompson, 1979). During paper chromatography of the digest, the methylated residue co-migrated with 2'-*O*-methyladenosine and, following elution, high-voltage paper electrophoresis at pH 3.5 confirmed that this residue was a derivative of adenosine. Finally, failure of borate to mobilize it during high-voltage paper electrophoresis at pH 9.3 confirmed the absence of *cis*-hydroxyl groups on the pentose moiety. We concluded that the methylated residue introduced into *S. coelicolor* rRNA by *S. actuosus* extract was 2'-*O*-methyladenosine.

DISCUSSION

The present data clearly establish that nosiheptide and thiostrepton act similarly on bacterial ribosomes, although the latter drug binds with greater affinity to what may be a common target site. Thus, both drugs act on the 50S ribosomal subunit and both bind firmly to the complex of 23S rRNA with ribosomal protein L11. Moreover, ribosomal binding of thiostrepton can be reduced to undetectable levels by the introduction into 23S rRNA of a single methyl group catalysed by an enzyme present in organisms which produce either of these drugs. Presumably, such methylation occurs at a location physically adjacent to the site(s) at which nosiheptide and thiostrepton bind, although their precise relationship remains to be determined. It is also evident that these events occur in or near that region of the A site on the 50S ribosomal subunit into which factors EF-Tu and EF-G bind alternately during protein synthesis and in which GTP is hydrolysed (for a discussion, see Cundliffe, 1980). Moreover, this same ribosomal domain is implicated in the binding and action of stringent factor (Wagner & Kurland, 1980).

Our conclusion that nosiheptide belongs (together with siomycin, sporangiomycin and thiopeptin) to the thiostrepton group of antibiotics rests not only on similarities in the modes of action of these drugs but also on the common mechanism of self defence employed by the respective producing organisms. Presumably, specific methylation of 23S RNA perturbs the structure of the ribosome within the GTPase centre. The aim of future investigations will be to characterize such conformational changes.

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