Mechanism of action of purpuromycin

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Purpuromycin, an antibiotic active against both fungi and bacteria, shows different modes of action against these two kinds of micro-organisms; in *Candida albicans* it inhibits RNA synthesis, whereas in *Bacillus subtilis* protein synthesis is primarily affected, with DNA and RNA synthesis blocked at higher concentrations of the drug. In bacterial cell-free protein-synthesis systems, purpuromycin did not inhibit synthesis from endogenous mRNA (elongation of peptides initiated within the intact cell) but inhibited MS2-phage RNA-dependent protein synthesis (which requires initiation) by 50 % at 0.1 mg/l. Poly(U)-directed polyphenylalanine synthesis was 50 % inhibited by 20 mg of purpuromycin/l when added to a complete system; however, when purpuromycin was preincubated with ribosomes dissociated into 30 S and 50 S subunits, the concentration for 50 % inhibition fell to 0.1 mg/l. By contrast, in a *C. albicans* cell-free system poly(U)-directed polyphenylalanine synthesis was partially inhibited only at 200 mg/l. Purpuromycin also inhibited polynucleotide synthesis *in vitro* in reactions using *Escherichia coli* or wheat-germ RNA polymerases or *E. coli* DNA polymerase I. We suggest that in bacteria the primary target of purpuromycin is on ribosomes and that its action precedes the elongation step of protein synthesis. The effect on nucleic acid synthesis in both fungi and bacteria may be due to interaction of purpuromycin with DNA.

INTRODUCTION

Purpuromycin is an antibiotic isolated from culture broth of *Actinoplanes ianthinogenes*. The structure of the molecule has been determined (Bardone *et al.*, 1974) and its spectrum of antibacterial and antifungal activity has been reported (Coronelli *et al.*, 1974). Purpuromycin is most active against Gram-positive bacteria [minimal inhibitory concn. (MIC) 0.06 mg/l for *Bacillus subtilis*] and somewhat less active against Gram-negative bacteria (MIC 4 mg/l for *Escherichia coli*) and fungi (MIC 1 mg/l for *Candida albicans*). It is bactericidal for *Staphylococcus aureus* but fungistatic for *C. albicans* (B. P. Goldstein, unpublished work). No data are available in the literature on the mechanism of action of purpuromycin in intact micro-organisms, although Rambelli *et al.* (1989) have reported that it inhibited cell-free protein synthesis both in *E. coli* and in eukaryotic systems (rabbit reticulocytes and *Artemia salina*). We have now studied the mode of action of purpuromycin in cultures of *B. subtilis* and *C. albicans*. Additional studies on its mechanism of action in cell-free protein-synthesis systems, as well as in RNA polymerase and DNA polymerase assays, are reported.

MATERIALS AND METHODS

Strains

For macromolecular-synthesis inhibition studies in intact cells, *B. subtilis* 566/1 (*thyA thyB*) and *C. albicans* SKF 2270 were used. The same strain of *C. albicans* and *E. coli* K12 HfrC were used as the sources of extracts for cell-free protein synthesis.

Chemicals

Purpuromycin was prepared at Lepetit Research Center, Gerenzano, Italy. It was dissolved in dimethylformamide at 100 times the final concentration; 1 % dimethylformamide was always added to controls.

All radioactive compounds were from The Radiochemical Centre, Amersham, Bucks., U.K. MS2-phage RNA, calf thymus DNA and *E. coli* RNA polymerase were from Boehringer, Mannheim, Germany. *E. coli* DNA polymerase I was from Promega, Madison, WI, U.S.A. All other chemicals were from Sigma, St. Louis, MO, U.S.A.

Inhibition of macromolecular synthesis

B. subtilis was grown at 37 °C to an A_{590} of 0.2 in Davies–Mingioli (1950) minimal medium supplemented with 2 % glucose, 0.4 % Difco casamino acids, 100 mg of asparagine/l and 1 mg of thymine/l. Macromolecular syntheses (DNA, RNA, protein and cell-wall peptidoglycan) were monitored by incorporation of the appropriate radioactive precursors: for DNA synthesis, [³H]-thymidine (2 µCi/ml), with 2 mg of unlabelled thymidine/l; for RNA synthesis, [³H]uridine (1 µCi/ml, 10 mg/l); for protein synthesis, [³H]tryptophan (1 µCi/ml, 1 mg/l); for cell-wall peptidoglycan, [³H]N-acetylglucosamine (2 µCi/ml, 3.5 mg/l). Samples (0.1 ml) were taken at intervals up to 60 min of incubation in the presence or absence of purpuromycin and were precipitated with ice-cold 5 % (w/v) trichloroacetic acid (TCA). Samples for protein synthesis were heated for 10 min at 80 °C.

C. albicans was grown at 32 °C to an A_{590} of 0.6 in a defined medium containing 3.5 g of NH₄Cl/l, 1 g of KH₂PO₄/l, 0.5 g of MgSO₄/l, 0.1 g of CaCl₂/l, 0.1 g of NaCl/l, 3 % (v/v) glycerol, 0.1 % glucose, 0.2 % Difco casamino acids and 0.1 % inositol. We measured DNA, RNA, protein, glucan and chitin synthesis. Precursors used were: [³H]adenine for DNA and RNA (2 µCi/ml, with 0.1 mg of unlabelled adenine/l for DNA, and 1 µCi/ml, 2 mg/l for RNA); [³H]methionine for protein synthesis (5 µCi/ml); [³H]glucose for glucan (4 µCi/ml); [³H]N-acetylglucosamine for chitin synthesis (4 µCi/ml, 1.4 mg/l). Samples (0.1 ml) were taken at intervals. Incorporation of [³H]adenine into DNA was determined as alkali-stable radioactivity. TCA-precipitated ma-

Abbreviation used: TCA, trichloroacetic acid.

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Fig. 1. Effect of purpurosmycin on macromolecular synthesis in *B. subtilis*: (a) DNA; (b) RNA; (c) protein; (d) peptidoglycan

The arrow indicates time of addition of the antibiotic. Key: Δ , purpurosmycin (0.25 mg/l); $\circ$, untreated controls. All data are from a single experiment.

terial was incubated for 24 h at room temperature in 3% (w/v) NaOH and then acidified. Total TCA-precipitable radioactivity (without alkali treatment) is essentially in RNA. Samples for protein synthesis were heated for 10 min at 80 °C after TCA precipitation. Incorporation of [^3H]glucose into glucan and of [^3H]N-acetylglucosamine into chitin was determined as radioactivity in hot-alkali-insoluble material, as described by Bagueley *et al.* (1979); glucan-synthesis samples were also incubated with 0.5 unit of chitinase from *Streptomyces griseus* (18 h at 28 °C in 0.1 M-phosphate buffer, pH 6.0). For 5 min incorporation experiments, labelled precursors were used at 1 μCi in 0.1 ml, with no addition of unlabelled precursors. In these experiments the A_{590} was 0.4 for *B. subtilis* and 1.0 for *C. albicans*.

Cell-free protein synthesis

E. coli ribosomes, supernatant protein-synthesis factors and crude initiation factors were prepared as described in Traub *et al.* (1971). Polyphenylalanine-synthesis assays, directed by poly(U), were performed in buffer A (30 mM-Tris/HCl, pH 7.9, 80 mM- NH_4Cl , 0.8 mM-ATP, 1 mM-GTP, 3 mM-dithiothreitol) plus 10 mM- MgCl_2 , 75 μg of poly(U), 0.8 μg of phenylalanine-specific tRNA from *E. coli*, 0.6 μCi of [^3H]phenylalanine (sp. radioactivity 25 Ci/mmol), 3 pmol of NH_4Cl -washed ribosomes and 2 μg of

supernatant factors. In some experiments preincubation at low MgCl_2 concentration was performed, as for MS2-phage RNA. Reactions were started by addition of poly(U). For MS2-phage-RNA-directed protein synthesis, 3 pmol of NH_4Cl -washed ribosomes and 4 μg of crude initiation factors were preincubated on ice for 30 min in buffer A plus 0.2 mM- MgCl_2 . The assay was run in buffer A plus 5 mM- MgCl_2 , 80 μg of *E. coli* total tRNA, 19 amino acids (400 pmol each), 1 μCi of [^3H]valine (sp. radioactivity 40 Ci/mmol), 0.4 mM citrovorum factor and 8 μg of MS2-phage RNA. Reactions were started with MS2-phage RNA. Synthesis from endogenous mRNA was performed in the same conditions, but with unwashed non-preincubated ribosomes. Reactions were started by addition of supernatant protein-synthesis factors. All reactions were performed at 30 °C for 30 min, in a final volume of 0.1 ml.

Cell-free protein-synthesis extracts from *C. albicans* were prepared as described in Feinberg & McLaughlin (1988). Briefly, protoplasts were lysed with a Dounce homogenizer (20 strokes in ice) and centrifuged at 30000 g for 30 min at 4 °C. The supernatant was collected and centrifuged at 100000 g for 3 h at 2 °C. The supernatant from the second centrifugation (S-100), containing ribosome subunits and supernatant factors, was dialysed against 30 mM-Tris/HCl, pH 7.9, containing 50 mM- NH_4Cl ,

6 mM-MgCl₂, 2 mM-dithiothreitol and 0.25 mM-phenylmethane-sulphonyl fluoride. Poly(U)-directed assays were run at 21 °C for 60 min in the same buffer as for bacterial poly(U)-directed assays; we used total tRNA from baker's yeast instead of from *E. coli*. Reactions were started by addition of poly(U). In all cell-free experiments, purpuromycin was preincubated for 30 min on ice with ribosomes and protein-synthesis factors either in the reaction buffer or in buffer A plus 0.2 mM-MgCl₂. All samples were precipitated with cold TCA to a final concentration of 5% and heated for 10 min at 80 °C.

RNA polymerase and DNA polymerase assays

RNA polymerase assays were run for 15 min at 37 °C as described by Gross *et al.* (1976); we used 0.5 unit of *E. coli* RNA polymerase or 1 unit of wheat-germ RNA polymerase. DNA polymerase assays were run for 30 min at 37 °C as described by Litman (1968); 0.3 unit of enzyme was used. The reactions were started by adding 1.25 µg of calf thymus DNA and terminated by addition of cold 5% TCA.

Determination of radioactivity

TCA-precipitated material was collected on glass-fibre filters by using a cell harvester (LKB), and radioactivity was determined in a Beta-plate scintillation counter (Pharmacia).

RESULTS

Inhibition of macromolecular synthesis in intact cells

When *B. subtilis* was exposed to 0.25 mg of purpuromycin/l, protein synthesis was immediately and completely blocked and DNA synthesis was partially inhibited (Fig. 1). Synthesis of RNA and peptidoglycan was affected only later. We then compared the concentration required to inhibit different macromolecular syntheses by exposing *B. subtilis* for 5 min to different concentrations of purpuromycin. Protein synthesis was almost completely inhibited at 0.2 mg/l, whereas significant inhibition of DNA, RNA and peptidoglycan synthesis occurred at 1 mg/l or higher (Fig. 2). Purpuromycin thus appears to inhibit protein synthesis preferentially in *B. subtilis*, although at higher concentrations it can affect other macromolecular syntheses.

In *C. albicans*, RNA synthesis was immediately blocked by 30 mg of purpuromycin/l, the minimal concentration that inhibits yeast growth in the conditions in which the experiment was performed; there was little effect on other syntheses (Fig. 3). When higher concentrations of purpuromycin were tested in 5 min experiments, no inhibition of protein, glucan or chitin synthesis was observed with up to 100 mg of the antibiotic/l; DNA synthesis was inhibited by 50% at 50 mg of purpuromycin/l and RNA synthesis at 20 mg/l (results not shown).

Cell-free protein-synthesis assays

In the *E. coli* system, 20 mg of purpuromycin/l inhibited poly(U)-directed polyphenylalanine synthesis by 50%, in agreement with previous observations by Rambelli *et al.* (1989), whereas protein synthesis from endogenous mRNA was unaffected by up to 200 mg of the antibiotic/l (Fig. 4). Both of these syntheses occur without the participation of initiation factors and involve only the elongation step of protein synthesis. In poly(U)-directed synthesis, correct initiation is by-passed (Schlessinger *et al.*, 1967). Translation from endogenous mRNA mainly reflects run-off of ribosomes on pre-existing polysomes (Gierer, 1963; Tai *et al.*, 1973). This is illustrated by the time course of protein synthesis in the endogenous mRNA system. During incubation for 30 min at 30 °C, 90% of the incorporation occurred within the first 10 min. This differs from the poly(U)

Fig. 2. Effect of different concentrations of purpuromycin on macro-molecular synthesis in *B. subtilis*

Values are expressed as percentages of synthesis in untreated controls: DNA (○; 2637 c.p.m.), RNA (□; 5825 c.p.m.), protein (◇; 3529 c.p.m.), peptidoglycan (△; 5485 c.p.m.). All data are from a single experiment.

system (and the MS2 system discussed below) in which incorporation was linear for at least 30 min (results not shown).

Because the inhibitory concentrations in both the poly(U) and the endogenous mRNA systems were much higher than those which inhibited protein synthesis in intact *B. subtilis*, it was likely that elongation was not the step of protein synthesis that is primarily affected by purpuromycin. MS2-phage RNA-dependent protein synthesis was used as a model of an initiation-dependent system. As shown in Fig. 5, purpuromycin inhibited MS2-RNA-directed protein synthesis by 50% at 0.1 mg/l. In this assay, ribosomes were preincubated with crude initiation factors in 0.2 mM-MgCl₂, to favour dissociation of ribosomes into 30 S and 50 S subunits (Traub *et al.*, 1971), and purpuromycin was added at this step. Poly(U)-directed synthesis became equally sensitive to purpuromycin when the antibiotic was preincubated with ribosomes under the same conditions, but without initiation factors. When polyribosomes were preincubated in 0.2 mM-MgCl₂, synthesis from endogenous mRNA was decreased by about 80%; the concentration of purpuromycin that inhibited the residual synthesis by 50% remained 200 mg/l (Fig. 5).

Since Rambelli *et al.* (1989) had reported that purpuromycin inhibited cell-free protein synthesis in *Artemia salina* and in rabbit reticulocyte systems, as well as in *E. coli*, we investigated the effect of purpuromycin on poly(U)-directed polyphenylalanine synthesis in a *C. albicans* cell-free system (Fig. 6). Slight inhibition was seen only with 200 mg of purpuromycin/l, which was preincubated with ribosomes in 0.2 mM-MgCl₂, the condition in which 0.1 mg of purpuromycin/l strongly inhibited bacterial cell-free protein synthesis.

RNA and DNA polymerase assays

The observation that purpuromycin primarily inhibited RNA synthesis in *C. albicans* and that it affected DNA and RNA synthesis in *B. subtilis* (although at concentrations higher than those inhibiting protein synthesis) suggested that the drug could interact with DNA. We therefore investigated its effect on

Fig. 3. Effect of purpuromycin on macromolecular synthesis in *C. albicans*: (a) DNA; (b) RNA; (c) protein; (d) glucan; (e) chitin

The arrow indicates time of addition of the antibiotic. Key: Δ , purpuromycin (20 mg/l); $\circ$, untreated controls. All data are from a single experiment.

DNA-dependent polynucleotide synthesis using *E. coli* RNA polymerase and DNA polymerase I and wheat-germ RNA polymerase. Purpuromycin inhibited all three enzymes by 50% at 10 mg/l (Fig. 7). A summary of the results of all inhibition studies is given in Table 1.

DISCUSSION

In experiments with intact *B. subtilis*, protein synthesis appeared to be the primary target of purpuromycin, although inhibition of nucleic acid synthesis was observed at higher concentrations of the antibiotic (Figs. 1 and 2). In order to understand the mechanism of inhibition of protein synthesis by purpuromycin, we performed inhibition experiments in different cell-free protein-synthesis systems, using MS2-phage RNA, poly(U) and endogenous mRNA. The MS2-directed system requires faithful initiation of protein synthesis, with dissociation of ribosomes into 30 S and 50 S subunits and formation of an initiation complex (Iwasaki *et al.*, 1968; Leffler & Szer, 1973). As poly(U) does not contain an initiation codon, synthesis from

poly(U) is performed at a Mg^{2+} concentration at which normal initiation is by-passed. The source of endogenous mRNA was a ribosome preparation in which cell-free protein synthesis is mainly elongation of peptide chains which were initiated on polysomes within the intact bacteria (Tai *et al.*, 1973).

In the initiation-independent endogenous-mRNA and poly(U) systems, purpuromycin inhibition occurred only at concentrations much higher than those inhibiting whole cells (Rambelli *et al.*, 1989, and Fig. 4). We have now shown that if ribosomes are preincubated with the antibiotic under conditions in which they dissociate into 30 S and 50 S subunits, both poly(U)-directed and MS2-phage-RNA-directed protein synthesis are inhibited at low antibiotic concentration (Fig. 5). As protein-synthesis initiation factors were added only to the MS2 system, we conclude that purpuromycin does not inhibit an initiation factor or prevent ribosome-initiation-factor interaction. It seems likely that dissociation of 70 S ribosomes exposes a purpuromycin-binding site and that, once bound, the antibiotic inhibits either reassociation of subunits or binding of mRNA or of the first aminoacyl-tRNA to the 30 S subunit. Purpuromycin probably failed to inhibit

Fig. 4. Effect of purpuromycin on poly(U)-directed polyphenylalanine synthesis (Δ) and endogenous mRNA-directed protein synthesis ($\circ$) in an *E. coli* cell-free system

Untreated controls incorporated 11.4 pmol of [3 H]phenylalanine [poly(U) system] and 0.25 pmol of [3 H]valine (endogenous mRNA). These experiments were performed three times with similar results. The data are from a typical experiment.

Fig. 5. Effect of preincubation in 0.2 mM-MgCl₂ on purpuromycin activity in cell-free systems

Controls incorporated 0.96 pmol of [3 H]valine (MS2 RNA; $\circ$), 9.6 pmol of [3 H]phenylalanine [poly(U); Δ] and 0.06 pmol of [3 H]valine (endogenous mRNA; $\square$). These experiments were performed twice with similar results. Data are from a typical experiment.

endogenous mRNA-directed protein synthesis after preincubation in 0.2 mM-MgCl₂ because the small amount of residual synthesis was from undissociated polyribosomes.

Although secondary to protein-synthesis inhibition in bacteria, inhibition of nucleic acid synthesis appears to be the primary mechanism of action of purpuromycin in fungi. In *C. albicans* RNA synthesis was the primary target (Fig. 3), whereas protein synthesis remained unaffected both in intact cells and in cell-free systems (Figs. 3 and 6), even at high purpuromycin concentrations. For this reason, we studied the effect of purpuromycin

Fig. 6. Effect of purpuromycin on poly(U)-directed polyphenylalanine synthesis in a *C. albicans* system

The untreated control incorporated 1.8 pmol of [3 H]phenylalanine. The experiment was performed twice with similar results. Data are from a typical experiment.

Fig. 7. Purpuromycin inhibition of DNA polymerase and RNA polymerase reactions

Untreated controls incorporated: *E. coli* DNA polymerase I (Δ), 1.4 pmol of [3 H]TMP; *E. coli* RNA polymerase ($\circ$), 8.2 pmol of [3 H]UMP; wheat-germ RNA polymerase ($\square$), 5.0 pmol of [3 H]-UMP. Each point is the mean of a duplicate assay.

on different DNA-dependent polymerases. Purpuromycin (10 mg/l) inhibited *E. coli* RNA polymerase and DNA polymerase I and wheat germ RNA polymerase by 50% (Fig. 7), suggesting that the drug may also interact with DNA. γ -Rubromycin, an antibiotic which has a chemical structure similar to that of purpuromycin, has also been reported to inhibit some polymerases (Goldman *et al.*, 1990); it inhibited mammalian DNA polymerase α by 50% at 3.2 mg/l. The concentrations of purpuromycin required to inhibit polymerase reactions, although close to those inhibiting RNA synthesis in *C. albicans*, were much higher than those which inhibited DNA and RNA synthesis in intact bacteria. It is possible that, in intact micro-organisms, an interaction between purpuromycin and DNA may affect other

Table 1. Summary of inhibitory activity of purpuromycin

Abbreviations: IC₅₀, concentration producing 50% inhibition; N.D., not determined.

(a) Intact-cell assays		
	<i>B. subtilis</i>	<i>C. albicans</i>
Purpuromycin concn. (mg/l)	0.25	20
Synthesis primarily affected	Protein	RNA
(b) Cell-free protein synthesis		
	IC ₅₀ (mg/l)	
Template	<i>E. coli</i>	<i>C. albicans</i>
MS2-phage RNA	0.1	N.D.
Endogenous mRNA	200	N.D.
Poly(U)	20	N.D.
Poly(U); dissociated ribosomes	0.1	200
(c) Enzyme assays		
	IC ₅₀ (mg/l)	
	<i>E. coli</i>	Wheat germ
RNA polymerase	10	10
DNA polymerase I	10	N.D.

enzymes involved in DNA and RNA synthesis (e.g. topoisomerases), at lower concentrations.

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