Inhibition of Leucyl-Transfer Ribonucleic Acid Synthetase in *Bacillus subtilis* by Granaticin

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The bacteriostatic effect of low concentrations of the antibiotic granaticin on *Bacillus subtilis* is relieved by the addition of leucine to the growth medium. In cells treated with granaticin, aminoacylation of leucine tRNA is specifically decreased, but the content of free leucine is not. It is concluded that granaticin interferes with the charging process of leucine tRNA in *B. subtilis* leading to leucine auxotrophy.

Granaticin was isolated from a strain of *Streptomyces olivaceus* by Corbaz *et al.* (1957). Its structure was clarified by Keller-Schierlein *et al.* (1968). The antibiotic is active against *Bacillus subtilis* but not against the Gram-negative *Escherichia coli*. We present evidence that the primary effect of granaticin leading to bacteriostasis of *B. subtilis* is interference with the aminoacylation of tRNA^{Leu}. In granaticin-treated cells the degree of aminoacylation of tRNA^{Leu} is drastically decreased, whereas the pool of free leucine is not, but is rather increased. To our knowledge, granaticin is only the second antibiotic to be found, borrelidin being the first (Hütter *et al.*, 1966; Nass & Hasenbank, 1970), that inhibits protein synthesis by reacting with an aminoacyl-tRNA synthetase.

Materials and Methods

Materials

Granaticin A (the algycone of granaticin B) was used throughout this investigation. It was supplied in crystalline form by Dr. H. Zähler, Tübingen University, Tübingen, W. Germany, and used without further purification. L-Amino acids were obtained from Boehringer, Mannheim, W. Germany. Salts for buffers (reagent grade) were from E. Merck, Darmstadt, W. Germany. [$G-^3H$]Uridine (6.4 mCi/mmol), [$U-^{14}C$]phenylalanine (522 mCi/mmol), [$U-^{14}C$]leucine (348 mCi/mmol), [$U-^{14}C$]isoleucine (342 mCi/mmol), [$U-^{14}C$]arginine (336 mCi/mmol), [$Me-^{14}C$]methionine (56 mCi/mmol), [$U-^{14}C$]valine (280 mCi/mmol) and [$6-^3H$]thymidine (5 Ci/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K.

Methods

Culture conditions. *B. subtilis* W23 was grown in Tris-buffered glucose-salt medium (Nakada, 1965)

at 37°C on a rotatory shaker. Growth of the culture was followed photometrically at 578 nm with an Eppendorf photometer. All experiments were carried out during steady exponential growth, starting with overnight cultures which were diluted to an absorbance of about 0.05 with fresh pre-warmed medium.

Measurement of RNA and protein synthesis. The incorporation of [3H]uridine and [^{14}C]phenylalanine into acid-precipitable material was followed in dual-labelled cultures (for details see Figure legends). The precipitated samples (usually 0.1 ml) were collected on Sartorius membrane filters, washed, dried and counted in 6 ml of toluene [containing 0.5% 2,5-diphenyloxazole, and 0.03% 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene] in a Packard liquid-scintillation spectrometer. In dual-label experiments the second channel (^{14}C) was adjusted to be free from tritium counts. The spill-over of ^{14}C into the tritium channel (18%) was not corrected for because of a label ratio of $^3H/^{14}C$ of about 10:1.

Determination of the content of free amino acids. A sample (30 ml) of the culture (E_{578} 0.3; about 10 mg wet weight) was quickly centrifuged without chilling. The pelleted cells were suspended in 1 ml of ice-cold water. Amino acids were extracted twice with a mixture of methanol and chloroform (2:1, v/v). The aqueous phases were combined, concentrated to dryness, resuspended in 0.5 ml of citrate buffer (Spackman *et al.*, 1958) and applied directly to the column of a Beckman amino acid analyser.

Degree of aminoacylation of tRNA *in vivo*. The method of Folk & Berg (1970b) as modified by Lewis & Ames (1972) was used except that a crude enzyme preparation was used as a source of aminoacyl-tRNA synthetase. The procedure described for *E. coli* and *Salmonella typhimurium* was found to be applicable to *B. subtilis*. The method is based on a quick acidification of the culture with trichloroacetic acid to conserve the ester bond between tRNA and amino acid. Transfer RNA is extracted by sonification in the

presence of phenol. In one-half of the extract the ribose of the uncharged tRNA is oxidized by periodate. This is followed by a discharging of the aminoacyl-tRNA in both halves of the extract. In both portions the tRNA is then charged with labelled amino acids. The percentage of tRNA-acceptor activity resistant to periodate oxidation reflects the degree of aminoacylation *in vivo*.

Results

Bacteriostasis, reversibility and bacteriolysis

Granatacin (Fig. 1) has a bacteriostatic effect on *B. subtilis* at concentrations of 0.1–0.5 $\mu\text{g/ml}$ (Ogilvie, 1970). Cells remain viable even after several hours of incubation with granatacin. Cells treated with low (0.1–0.6 $\mu\text{g/ml}$) concentrations of granatacin recover spontaneously after several hours by a still unknown mechanism. Prolonged incubation with higher concentrations of granatacin causes lysis of *B. subtilis* cells.

Fig. 1. Structural formula of granatacin A

Table 1. Inhibition of RNA and DNA accumulation in *B. subtilis* by granatacin

Exponentially growing cultures (10 ml, E_{578} about 0.3) were treated with granatacin or used as controls. After 5 min [³H]uridine (0.1 $\mu\text{Ci/ml}$; 6 $\mu\text{Ci/mmol}$) or [³H]-thymidine (0.4 $\mu\text{Ci/ml}$; 50 $\mu\text{Ci/mmol}$) was added. At intervals of 5 min, samples (2 ml) were pipetted into ice-cold HClO_4 (0.2 ml; 5 M). After centrifugation and washing of the sediments with diluted HClO_4 (0.5 M) the incorporation of radioactivity into acid-insoluble material was measured and taken as RNA accumulation and DNA accumulation respectively. The degree of inhibition was calculated from the kinetics of incorporation in the granatacin-treated cultures relative to the untreated controls.

Granatacin ($\mu\text{g/ml}$)	% inhibition	
	RNA	DNA
0.1	70	30
0.2	100	60
0.5	100	85

Inhibition of the synthesis of macromolecules

The net synthesis of DNA, RNA and protein was measured under the influence of granatacin at increasing concentrations. The rationale was that the most sensitive process of the cell might indicate the primary action of granatacin leading to bacteriostasis. The incorporation of labelled uridine, phenylalanine and thymidine is always inhibited simultaneously, but RNA synthesis seems to be affected predominantly (Fig. 2 and Table 1). Direct interference with RNA synthesis has been suggested, but various approaches to confirm this assumption were unsuccessful. The activity of DNA-dependent RNA polymerase *in vitro* was not affected by granatacin (G. Hartmann, personal communication). Intercalation or binding of granatacin to DNA was ruled out by co-sedimentation experiments. The 'melting' curve of DNA was not altered in the presence of the drug. Addition of DNA or RNA to the culture medium does not decrease the inhibitory effect of granatacin on growing cells. Further investigation clearly showed that the inhibition of RNA accumulation is a consequence of the inhibition of protein synthesis (Ogilvie *et al.*, 1975b).

Leucine auxotrophy of granatacin-treated cells

B. subtilis cultivated in broth medium needs significantly higher concentrations of granatacin for inhibition than do cultures grown in glucose-salt medium. The addition of casein hydrolysate or a mixture of amino acids to the minimal medium lowers or even prevents the inhibition. This gave rise to the question of whether a single amino acid or a mixture of amino acids is responsible for the relieving effect. Since we must consider the possibility that a concerted action of the amino acids is involved in the relieving mechanism, we have tried to reverse the effect of granatacin by adding various mixtures of amino acids in which a single amino acid was always eliminated from the complete mixture of the 19 essential amino acids. Cysteine, which is not necessary for the relief, was omitted from all mixtures to avoid reaction with the drug. The results obtained from measuring growth and RNA and protein accumulation were equivalent and are presented only representatively. Leucine was found to be obligatory for the prevention of the inhibitory effect of granatacin (Fig. 3), but the other amino acids were not necessary (Fig. 3). Up to a concentration of 0.2 μg of granatacin/ml, leucine alone (20 μM) is able to prevent the inhibition of RNA and protein synthesis (Fig. 4). At slightly higher concentrations of granatacin, leucine produces only partial relief, even when added at a much higher concentration (2 mM). With increasing concentrations of granatacin, methionine and other amino acids in combination with leucine become

Fig. 2. Inhibition of RNA (a) and protein (b) accumulation in *B. subtilis* by granaticin

An exponentially growing culture was labelled with [^3H]uridine (5 $\mu\text{Ci/ml}$; 62.5 $\mu\text{Ci/mmol}$) (a) and [^{14}C]phenylalanine (0.25 $\mu\text{Ci/ml}$; 2 mCi/mmol) (b). Granaticin was added (indicated by arrow) at E_{578} of the culture of about 0.250. Samples (50 μl) were withdrawn and the incorporation of radioactivity into acid-insoluble material was determined. ●, Control; □, granaticin-treated (0.1 $\mu\text{g/ml}$); ○, granaticin-treated (0.15 $\mu\text{g/ml}$).

necessary for growth. We followed the primary auxotrophy for leucine, because from the data presented it was suggested that granaticin primarily causes intracellular starvation for leucine.

Content of leucyl-tRNA^{Leu} in granaticin-treated cells

The antagonism between granaticin and externally added leucine does not prove the hypothesis of internal leucine starvation. We therefore determined the degree of aminoacylation of tRNA^{Leu} in normally growing and in granaticin-treated cells. The aminoacylation of tRNA^{Leu} was specifically and drastically

Fig. 3. Leucine auxotrophy of *B. subtilis* treated with granaticin

Granaticin (0.3 $\mu\text{g/ml}$) was added [arrow (2)] to an exponentially growing culture of *B. subtilis* monitored photometrically at 578 nm. About 20 min before the addition of the drug, mixtures of amino acids were pipetted in [arrow (1)]. ●, Control; ○, granaticin alone; □, granaticin plus standard mixture of 19 amino acids (alanine, arginine, asparagine, aspartate, glutamate, glutamine, glycine, histidine, leucine, isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine; each at a final concentration 1 mM); △, granaticin plus 18 amino acids (standard mixture minus leucine).

decreased in granaticin-treated cells (Table 2). This confirms the assumption that uncharged tRNA^{Leu} is the crucial factor in growth inhibition.

Content of leucine in the free pool

Two possible reasons for the requirement of leucine for the growth of granaticin-treated cells were considered: (1) an internal lack of leucine; (2) the failure of leucyl-tRNA synthetase to charge tRNA^{Leu} with its cognate amino acid. The intracellular pool of amino acids was determined after extraction and analysis. The content of leucine in granaticin-treated cells was not found to be decreased, but significantly increased (Table 3). Generally, the contents of amino acids were increased in granaticin-treated cells, probably as a consequence of the cessation of protein synthesis. Leucine and valine increased most markedly. Isoleucine, however, did not accumulate. We assume that the increase in valine and α -oxo-isovaleric acid might regulate the synthesis of isoleucine more strongly, in a manner similar to the

Fig. 4. Effect of leucine on RNA accumulation (a) and protein synthesis (b) in *B. subtilis* treated with granaticin

The dual-labelled culture was treated as described in the legend of Fig. 2. Leucine ($20 \mu\text{M}$) was added simultaneously with granaticin ($0.15 \mu\text{g/ml}$) at the time indicated by the arrow. ●, Control; ○, granaticin; □, granaticin plus leucine.

regulation observed in *E. coli* (Rodwell, 1969; Umbarger, 1969).

Discussion

Granaticin causes reversible suppression of growth and protein and RNA synthesis in *B. subtilis*. At low concentrations the inhibition can be overcome by the addition of an excess of leucine. Aminoacylation of tRNA^{Leu} was decreased in granaticin-treated cells,

Table 2. Aminoacylation of tRNA in *B. subtilis* treated with granaticin

Two exponentially growing cultures (500 ml; E_{578} about 0.5) were either treated with $0.2 \mu\text{g}$ of granaticin/ml for 8 min or used as a control. Growth was stopped by the addition of trichloroacetic acid (final concentration 5%). Extraction of tRNA, periodate treatment and the determination of the degree of aminoacylation are described in the Materials and Methods section.

Amino acid	Percentage aminoacylation	
	Control	Granaticin-treated
Leucine	85	25
Isoleucine	100	90
Valine	100	90
Phenylalanine	80	85
Arginine	85	90
Methionine	100	100

Table 3. Intracellular amino acid concentration in *B. subtilis* treated with granaticin

Extraction and determination of amino acids were as described in the Materials and Methods section.

Amino acid	Amino acid concentration (nmol/20 mg wet wt.)	
	Control	Granaticin-treated
Leucine	9	24
Isoleucine	3	3
Valine	12	27
Glycine	12	16
Alanine	24	48
Aspartic acid	33	53
Tyrosine	6	9
Phenylalanine	18	26

whereas the content of leucine was not. It is concluded that granaticin causes auxotrophy of leucine by interfering with leucyl-tRNA synthetase. A similar mechanism has been described for borrelidin which interferes with threonyl-tRNA synthetase (Hütter *et al.*, 1966). Several mutants with an altered aminoacyl-tRNA synthetase which are also auxotrophs for their cognate amino acids have been found (Folk & Berg, 1970a,b; Iaccarino & Berg, 1971; Straus & Ames, 1973).

Granaticin inactivates leucyl-tRNA synthetase from *B. subtilis* in a crude enzyme preparation. The molecular mechanism of inhibition is presently not understood. Further, we would like to know, by comparing various aminoacyl-tRNA synthetases, whether the specificity observed *in vivo* can be reflected *in vitro*.

The crucial event leading to bacteriostasis of granaticin-treated cells is the failure to charge tRNA^{Leu}. Uncharged tRNA is not the consequence of inhibition of protein synthesis, since we and others (Cassio and Mathien, 1974; Glazier & Schlessinger, 1974) have found, on the contrary, an increase in aminoacylation during inhibition of protein synthesis. The primary action of granaticin is the decrease in protein synthesis. The concomitant inhibition of RNA synthesis must be the well-known regulatory phenomenon, the so-called stringent control of RNA synthesis in amino-acid-deprived bacteria (Neidhardt, 1966; Edlin & Broda, 1968).

Results obtained from experiments with a stringent and relaxed pair of *B. subtilis* (isogenic except for the *rel* gene), strongly support this assumption and exclude an additional effect of granaticin on RNA synthesis (Ogilvie *et al.*, 1975b). Interference by granaticin with oxidative phosphorylation can also be ruled out since the content of ATP increased in cells treated with granaticin (Ogilvie *et al.*, 1975b).

6-Amino-7-chloro-5,8-dioxoquinoline inhibits the growth of *E. coli* by a similar mechanism (Ogilvie *et al.*, 1975a). From this analogy we assume that the quinone ring component of granaticin might be responsible for its effect in *B. subtilis*. Preliminary studies with other quinones also confirm the assumption that a variety of bacteriostatic quinones have a common mode of action related to that described here.

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