The Regulation of Stable RNA Synthesis in the Blue-green Alga *Anacystis nidulans*: Effect of Leucine Deprivation and 5-Methyltryptophan Inhibition

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The expression of phenomena associated with the bacterial function controlling RNA synthesis was studied in leucine-deprived or 5-methyltryptophan-treated cultures of *Anacystis nidulans*. Both procedures retarded cell growth, RNA and protein accumulation, elicited the accumulation of high intracellular concentrations of guanosine 5'-diphosphate-3'-diphosphate (ppGpp) and guanosine 5'-triphosphate-3'-diphosphate (pppGpp), and promoted a regime of non-coordinate synthesis of stable and messenger RNA. The rate of polymerization of nascent RNA chains did not appear to be retarded in the growth-limited cultures.

INTRODUCTION

The RNA composition of *Anacystis nidulans* is regulated according to growth rate in a manner similar to that described for bacteria. Thus increase in growth rate produces organisms with larger mass and volume and which contain more RNA per cell (Mann & Carr, 1974). The control of RNA synthesis in *A. nidulans* deviates from that of heterotrophic prokaryotes in that the ratio of transfer RNA to ribosomal RNA does not alter with variation in growth rate (Mann & Carr, 1973). The proportions of the genome coding for the major RNA fractions and the relative rates of expression of stable and unstable RNA cistrons are comparable to those of *Escherichia coli* (Smith & Carr, 1977) and encourage the further comparison of the regulation of RNA synthesis in autotrophic and heterotrophic prokaryotes. The accumulation of the stable RNA species (rRNA and tRNA) in certain bacteria is under the control of a regulatory mechanism known as the RC function which is controlled indirectly by the intracellular supply of amino acids and restrictions placed upon the aminoacylation of tRNA (Maaløe & Kjeldgaard, 1966; Eldin & Broda 1968). When aminoacylation of the tRNA pool is limited, either by a deficiency in one or more amino acids within the intracellular pool or by inhibition of the aminoacyl-tRNA synthetases, an idling reaction is initiated in the process of polypeptide chain elongation (Cashel & Gallant, 1969; Haseltine *et al.*, 1972). This reaction gives rise to two unusual guanosine nucleotides, guanosine 5'-diphosphate-3'-diphosphate (ppGpp) and guanosine 5'-triphosphate-3'-diphosphate (pppGpp) (Cashel, 1969; Cashel & Kalbacher, 1970).

In bacteria the effects of the RC function are primarily dependent upon the state of the aminoacyl-tRNA pool and not upon that of the amino-acid pool of the cell (Kaplan, 1969). These effects may be distinguished by the use of inhibitors of aminoacyl-tRNA synthetase activity in *E. coli* which prevent aminoacylation but do not deplete the amino-acid pools.

The tryptophan analogue 5-methyltryptophan has been used for this purpose (de Boer *et al.*, 1973). It inhibits aminoacylation of tryptophan-specific tRNA but is not incorporated into protein (Pardee, Shore & Prestidge, 1957; Pardee & Prestidge, 1958).

The present communication describes phenomena present in *A. nidulans* which are characteristic of the expression of the RC function in heterotrophic bacteria, and compares the elicitation of the phenomena through amino-acid deprivation of an auxotroph and by addition of an amino-acid analogue. The results suggest that the regulatory phenomena in *A. nidulans* is primarily elicited by reduced aminoacylation of tRNA.

METHODS

Organisms. These were *Anacystis nidulans* strain 625 (from The Culture Collection of Algae at the University of Texas, previously at the University of Indiana), and strain Leu201, a leucine-requiring auxotroph derived from strain 625. We are indebted to Dr W. Ford Doolittle for strain Leu201.

Media and growth conditions. Cultures were maintained in medium C (Kratz & Myers, 1955) modified by the addition of NaHCO_3 (1.0 g l^{-1}). Low-phosphate medium C was obtained by reducing the KH_2PO_4 concentration to $7.0 \times 10^{-5} \text{ M}$ and adding Tris to 0.4 g l^{-1} ; the pH was adjusted to 7.8 with 1 M-HCl . The leucine-requiring auxotrophic strain was grown in modified medium C supplemented with leucine ($5 \times 10^{-3} \text{ M}$). Cultures were maintained as previously described (Smith & Carr, 1977) or as 100 ml cultures in 250 ml conical flasks incubated at $35 \pm 0.5^\circ \text{C}$ in an orbital incubator at 84 rev. min^{-1} (L.H. Engineering, Stoke Poges, Buckinghamshire) aerated with air/ CO_2 (95:5, v/v) and illuminated by seven fluorescent tubes (Atlas Warm White, 13 W).

Experimental cultures were derived from 16 h cultures which had a mean generation time of 5 to 6 h and a turbidity (E_{750}) of 0.25 to 0.35. To estimate the effects of 5-methyltryptophan, cultures were divided into two: one part received 5-methyltryptophan to a final concentration of $100 \mu\text{g ml}^{-1}$; the other received no subsequent additions and was maintained as a growing control. To deprive cultures of strain Leu201 of leucine, exponential phase cultures were poured into cooled 250 ml bottles, centrifuged at 6°C and the pellet was washed in leucine-free medium. The procedure was repeated three times. Small volumes, especially cultures containing [^{32}P]orthophosphate were deprived of leucine by filtration on nitrocellulose filters (Sartorius membrane filter MF50, 25 mm diam., $0.46 \mu\text{m}$ pore size) followed by washing with 6 vols of prewarmed leucine-deficient medium. The final resuspension was in prewarmed medium. Control cultures were derived from material taken through the procedures and immediately resupplied with leucine ($5 \times 10^{-3} \text{ M}$). The former procedure took 20 to 30 min; the latter took about 15 min.

Growth was measured at 750 nm in a Cecil spectrophotometer (Cecil Instruments, Cambridge) using a 1 cm light path.

Radioisotope incorporation. The incorporation of [$2\text{-}^{14}\text{C}$]uracil, [$\text{G-}^3\text{H}$]tryptophan or [$1\text{-}^{14}\text{C}$]leucine (The Radiochemical Centre, Amersham) into acid-precipitable, and alkaline-stable acid-precipitable material was followed as described by Pigott & Midgley (1968). Glass fibre filters (25 nm, Whatman GF/C) were used in conjunction with a Nuclear Chicago Isocap 300 scintillation counter.

Isolation of phosphorylated nucleotides in formic acid extracts. Cultures grown in low-phosphate medium C were supplemented with [^{32}P]orthophosphate (specific radioactivity 5000 Ci g^{-1} , final concentration $50 \mu\text{Ci ml}^{-1}$; The Radiochemical Centre, Amersham) for at least one generation time before use. In experiments which required the removal of leucine from strain Leu201 cultures, [^{32}P]orthophosphate was included in the resuspension medium at the same specific radioactivity.

Samples (0.2 ml) were removed into ice-cold centrifuge tubes containing 0.1 ml of 98% (Analar) formic acid. After 20 min the mixtures were centrifuged (3000 g , 4°C , 10 min) and the upper portion of the supernatant was collected. Samples (10 μl) were applied to polyethyleneimine-cellulose plastic-backed thin-layer plates (cat. no. G-1440-PEI, $20 \times 20 \text{ cm}$; Schleicher and Schuell, Dassel, West Germany) and chromatographed as described by Cashel (1969) in $1.5 \text{ M-KH}_2\text{PO}_4$ pH 3.4. Radioactive material was located by autoradiography and these areas were cut from the plates and counted in a scintillation counter. The nucleotides ppGpp, pppGpp and GTP were identified from their R_f values as described by Cashel (1969). The validity of the technique for isolating ppGpp has been confirmed by Mann, Carr & Midgley (1975) by comparison with ppGpp isolated from *E. coli* and co-chromatographed with the *A. nidulans* extract in three separate systems and by two-dimensional chromatography.

RNA and protein estimation. Duplicate samples (5 ml) were treated essentially as described by Mann *et al.* (1975). The product of the NaOH digest was used to estimate RNA content by the method of Schneider (1957) and protein by the method of Lowry *et al.* (1951).

DNA-RNA hybridization. To determine accurately changes in the relative flux of labelled precursor into

Fig. 1. Effect of 5-methyltryptophan on the incorporation of amino-acid supplements into protein in *A. nidulans*. (a) Incorporation of L-[G-³H]tryptophan (20 mCi mmol⁻¹, 0.2 μ Ci ml⁻¹) into acid-precipitable material: 5MT (100 μ g ml⁻¹) was added at 30 min. (b) Effect of addition of DL-tryptophan on the incorporation of [1-¹⁴C]leucine (10 mCi mmol⁻¹, 0.2 μ Ci ml⁻¹) into acid-precipitable material in 5MT-treated cultures: 5MT (100 μ g ml⁻¹) was added at time 0, DL-tryptophan (20 μ g ml⁻¹) at 60 min. $\circ$, Growing control culture; $\bullet$, 5MT-treated culture; $\square$, 5MT-treated culture supplemented with tryptophan.

mRNA and stable RNA fractions the technique of coincident hybridization was employed (Midgley & Gray, 1971; Midgley & Smith, 1973). Experimental cultures were pulse-labelled with [³²P]orthophosphate and the growing control cultures were pulse-labelled with [5-³H]uracil as described by Smith & Carr (1977). ³²P-labelled RNA was cohybridized with ³H-labelled RNA at an overall DNA:RNA ratio of 5:1 to minimize stable RNA hybrid formation (Smith & Carr, 1977).

Estimations of RNA polymerization rates. The antibiotic rifampicin inhibits the initiation of new RNA chains while allowing completion of those already initiated. Using a technique devised by Pato & Von Meyenburg (1970), the time required to complete RNA synthesis after treatment with rifampicin was estimated. This value directly reflects the rate of polymerization of nascent RNA (Gray & Midgley, 1971) but does not depend upon the rate of incorporation of labelled precursor. The assumption was made that the addition of rifampicin to a final concentration of 200 μ g ml⁻¹ does not significantly alter RNA polymerization rates.

RESULTS

In comparing the effects of deprivation of a required amino acid from an auxotrophic strain, Leu201, and the addition of 5-methyltryptophan (5MT) to strain 625 of *A. nidulans*, we have sought to establish the major factor eliciting the phenomena described as being due to a reduced aminoacylation of tRNA. Thus the assumption that 5MT restricts growth by inhibiting tryptophan-specific aminoacyl-tRNA synthetases in *A. nidulans* is required. Figure 1 provides evidence to support this assumption. The incorporation of [G-³H]-tryptophan into trichloroacetic acid (TCA)-precipitable material in 5MT-treated cultures of strain 625 was inhibited compared with that in the growing control. The kinetics of incorporation were comparable to those of protein accumulation (Fig. 3a). The addition of DL-tryptophan (final concentration 20 μ g ml⁻¹) to 5MT-treated cultures did not significantly affect the incorporation of [1-¹⁴C]leucine into TCA-precipitable material (Fig. 1b).

Addition of 5MT to cultures of strain 625 or deprivation of leucine from cultures of strain Leu201 inhibited growth preventing both increases in cell size and cell division (Fig. 2). While leucine deprivation decreased the growth rate to 5 to 10% of that of the growing

Fig. 2. Inhibition of growth of *A. nidulans* by (a, b) addition of 5-methyltryptophan to cultures of strain 625, or (c, d) deprivation of leucine from cultures of strain Leu201. The arrows (in a, b) denote the addition of 5MT ($100 \mu\text{g ml}^{-1}$). $\circ$, Growing control culture; $\bullet$, inhibited culture.

control, 5MT addition had a more gradual effect, about 4 h being required. Adding leucine to leucine-deprived cultures of strain Leu201 allowed a rapid return to exponential growth.

Both procedures inhibited the accumulation of RNA and protein, reducing the rates of accumulation to between 10 and 15% of the growing control values. Again the inhibitory effect of 5MT (Fig. 3) was established more slowly than that of leucine deprivation. A similar pattern was shown in the accumulation kinetics of ppGpp. Both procedures induced the accumulation of ppGpp and pppGpp within the culture but leucine deprivation repeatedly effected maximum concentrations of the nucleotides in half the time required by 5MT treatment (Fig. 4).

The relative flux of [^{32}P]orthophosphate into stable and messenger RNA fractions during leucine deprivation and 5MT treatment was determined by the technique of coincident hybridization. Thus the proportion of pulse-labelled readily hybridizable RNA (at a DNA:RNA ratio of 5:1) in inhibited cultures may be directly compared with that in a growing control for each estimate made. Assuming the relative rate of synthesis of mRNA in steadily growing cultures to be 40% (Smith & Carr, 1977), the relative proportion in the inhibited samples may be expressed as a percentage providing corrections are made to account for the different specific radioactivities of the RNA samples. Figure 5 depicts the proportion of mRNA in pulse-labelled total cell RNA in leucine-deprived cultures. An increase from 40% to approximately 58% is noted. Similar though retarded effects were observed when 5MT was added to strain 625 cultures.

Providing a regulated degradation of nascent RNA is not involved, the regulation of RNA synthesis, which is a transcriptional event directed by a linear template, must depend on factors affecting either initiation, polymerization or termination of nascent RNA. A

Fig. 3. Effect of 5-methyltryptophan on (a) protein accumulation and (b) RNA accumulation in *A. nidulans*. The arrows denote the addition of 5MT ($100 \mu\text{g ml}^{-1}$). $\circ$, Growing control culture; $\bullet$, 5MT-inhibited culture.

Fig. 4. Accumulation of guanosine nucleotides in (a) cultures of *A. nidulans* strain Leu201 deprived of leucine and (b) cultures of *A. nidulans* strain 625 treated with 5-methyltryptophan ($100 \mu\text{g ml}^{-1}$) at time 0. $\circ$, ppGpp in inhibited cultures; $\bullet$, ppGpp in growing control cultures; $\square$, GTP in inhibited cultures; $\blacksquare$, GTP in growing control cultures; $\triangle$, pppGpp in inhibited cultures.

Fig. 5

Fig. 6

Fig. 5. Proportion of mRNA in pulse-labelled RNA in leucine-deprived cultures of *A. nidulans*. Strain Leu201 was resuspended at time 0. The first experimental sample was pulse-labelled immediately before commencing the centrifugation procedure. The results are expressed as a percentage of the total labelled RNA, assuming the value for the growing control (see text). $\circ$, Growing control culture; $\bullet$, leucine-deprived culture.

Fig. 6. Effect of leucine deprivation of *A. nidulans* strain Leu201 on the time taken to complete RNA synthesis after treatment with rifampicin: (a) growing control culture; (b) culture deprived of leucine for 30 min. Rifampicin ($200 \mu\text{g ml}^{-1}$) was added at time 0 simultaneously with $[2-^{14}\text{C}]\text{uracil}$ (54 mCi mmol^{-1} , $0.2 \mu\text{Ci ml}^{-1}$). $\circ$, Incorporation of ^{14}C radioactivity into DNA; $\bullet$, incorporation of ^{14}C radioactivity into RNA.

non-coordinate synthesis of stable and messenger RNA fractions (such as that described above) may be found in purine deprivation of *E. coli* (Varney, Thomas & Burton, 1970; Smith & Midgley, 1973). The times required to complete RNA synthesis after addition of rifampicin to growing and leucine-deprived cultures of strain Leu201 are compared in Fig. 6. The results suggest that neither polymerization nor termination events play a significant role in the regulatory phenomenon.

DISCUSSION

Leucine deprivation and 5MT treatment of *A. nidulans* cultures curtail growth, RNA and protein accumulation, promote accumulation of high intracellular concentrations of ppGpp

and pppGpp, and promote a non-coordinate synthesis of stable and messenger RNA. One may assess these results in two ways. The data support the concept of a mechanism regulating the extent and product of *A. nidulans* transcription, and suggest that the mechanism is elicited by reduced aminoacylation of tRNA rather than by reduction of amino-acid pools. The regulation appears to be directed at the initiation, rather than polymerization or termination, of nascent RNA chains. Secondly, the results may be compared with those from the extensive bacterial work (cf. Stent & Brenner, 1961; Fiil & Friesen, 1968; Cashel & Gallant, 1969; Cashel, 1969; Norris & Koch, 1972) which inspired this study. The similarity of the bacterial and blue-green algal phenomena imply that the suggested mechanism is akin to the bacterial RC function.

The accumulation of ppGpp during step-down procedures (Gallant, Margason & Finch, 1972; Mann *et al.*, 1975) has been attributed to reduced rates of turnover (Laffler & Gallant, 1974), whereas the accumulation during amino-acid deprivation results from increased rates of synthesis. The synthesis of ppGpp has been shown to be dependent upon a ribosome-associated protein known as 'stringent factor', the absence of which prevents ppGpp accumulation in *rel*⁻ mutants (Haseltine *et al.*, 1972). The accumulation of the guanosine nucleotides during leucine deprivation and 5-methyltryptophan inhibition of *A. nidulans* cultures indicates the 'RC^{str}' phenotype of this species and suggests the presence of a functional stringent factor.

The similarities between the bacterial and blue-green algal mechanisms are also observed during alleviation of the inhibitory effects and in the antagonistic effects of antibiotics. These will form the basis of a future communication.

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