UNFORMYLATED INITIATOR tRNA IS NOT A SIGNAL FOR THE STRINGENT CONTROL OF RNA SYNTHESIS

H.-H. Arnold and A. Ogilvie

Institut für Physiologische Chemie, Universität
Erlangen-Nürnberg, 3520 Erlangen, W.-Germany

Received November 11,1976

SUMMARY

When *Escherichia coli* MRE 600 or *Bacillus subtilis* W 23 are grown in glucose-salt medium supplemented with purines, thymidine and glycine, trimethoprim stops the synthesis of protein by causing a specific lack of methionyl-tRNA. The synthesis of RNA is simultaneously restricted by the stringent control mechanism. Guanosine tetraphosphate (ppGpp) largely accumulates. The addition of methionine abolishes the level of ppGpp and relieves the inhibition of RNA synthesis. The aminoacylation of methionine-specific tRNAs was found to be completely restored. The methionyl-$tRNA_f^{Met}$ however does not become formylated. These results indicate that unformylated initiator tRNA is not a sufficient condition for the accumulation of ppGpp and the onset of stringent control.

INTRODUCTION

The signal for the stringent control of RNA synthesis in bacteria deprived for an amino acid (1,2) is an uncharged tRNA in the acceptor site of the ribosome (3,4,5). Guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) largely increase under those conditions (6,7). Guanosine tetraphosphate has been suggested to be involved in the mechanism of stringent control (7,8,9). From studies with inhibitors of initiation of protein synthesis it has been assumed that a coupling exists between ppGpp formation and an idling reaction of one of the initiation steps (10).

Trimethoprim inhibits dihydrofolate reductase in bacteria and causes

ABBREVIATIONS

ppGpp, guanosine 3'-diphosphate-5'-diphosphate; pppGpp, guanosine 3'-diphosphate-5'-triphosphate; trimethoprim (TRM), 2,4-diamino-5(3',4',5'-trimethoxybenzyl)pyrimidine.

an intracellular depletion of purines, thymidine, glycine and methionine (11,12). Furthermore it prevents the formylation of initiator $tRNA_f^{Met}$ (13). From experiments with trimethoprim it has been suggested that unformylated initiator tRNA provides a condition for the accumulation of ppGpp in *E. coli* and for the onset of stringent control (14,15). In those studies, however, the degree of aminoacylation of $tRNA^{Met}$ and the degree of formylation of methionyl-$tRNA_f^{Met}$ had not been determined and therefore could not be correlated with the phenomenon of stringent control. We have measured these two parameters in *E. coli* and *B. subtilis* treated with trimethoprim. The results show that the stringently controlled strains of *E. coli* or *B. subtilis* are able to accumulate RNA when methionyl-$tRNA_f^{Met}$ is not formylated.

MATERIALS AND METHODS

Chemicals.

Trimethoprim (TRM) was a gift from Hoffman-La Roche AG, Grenzach, Germany. L-amino acids, purine nucleosides and thymidine were obtained from Boehringer, Mannheim, Germany. Reagents, solvents and PEI-cellulose thin-layer plates were from Merck AG, Darmstadt, Germany. The cation exchange resin AG 50W-X12 (H^+-form) was purchased from Bio-Rad Laboratories, Richmond, California. [^{3}H]uridine (6.4 Ci/mmol), [^{14}C]phenylalanine (522 mCi/mmol) and [^{32}P]orthophosphate (carrier free) were from the Radiochemical Centre Amersham, England.

Growth conditions.

Escherichia coli MRE 600 and *Bacillus subtilis* W23 were grown as described previously (16). For labeling experiments with [^{32}P]orthophosphate the medium contained 5×10^{-4}M KH_2PO_4 and was buffered with TRIS (0.1 M; pH = 7). The media were supplemented with guanosine, adenosine, thymidine (100 µg/ml each) and all common amino acids (100 µg/ml each) except methionine. Growth was followed photometrically at 578 nm. All experiments were carried out with exponentially growing cells.

Determination of ppGpp.

The incorporation of [^{32}P]orthophosphate into guanosine tetraphosphate was measured as described by Cashel (7).

Synthesis of RNA and protein.

Incorporation of [^{3}H]uridine (5 µCi/ml; 62.5 Ci/mol) and [^{14}C]phenylalanine (0.25 µCi/ml; 2 Ci/mol) into trichloroacetic acid insoluble material was used to measure the syntheses of RNA and protein respectively. Details are given in the legend of figure 2 and in reference 17.

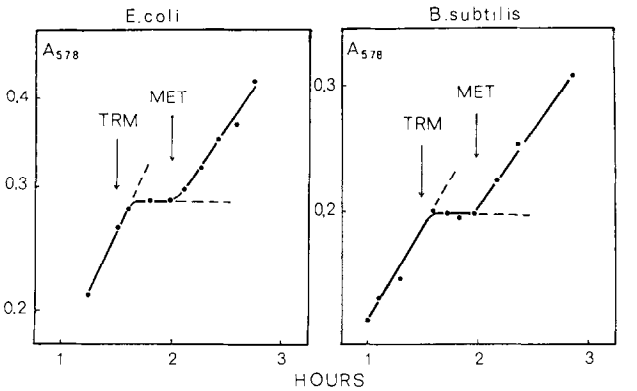

Figure 1. Inhibition of growth of *E. coli* MRE 600 and *B. subtilis* W23 by trimethoprim and the relieving effect of methionine.
Cultures of *E. coli* or *B. subtilis*, exponentially growing in a supplemented medium (as described under Materials and Methods), were treated with trimethoprim (TRM; 50 μg/ml). Methionine was added 30 min later to a final concentration of $1x10^{-4}$M. Growth was monitored photometrically at 578 nm.

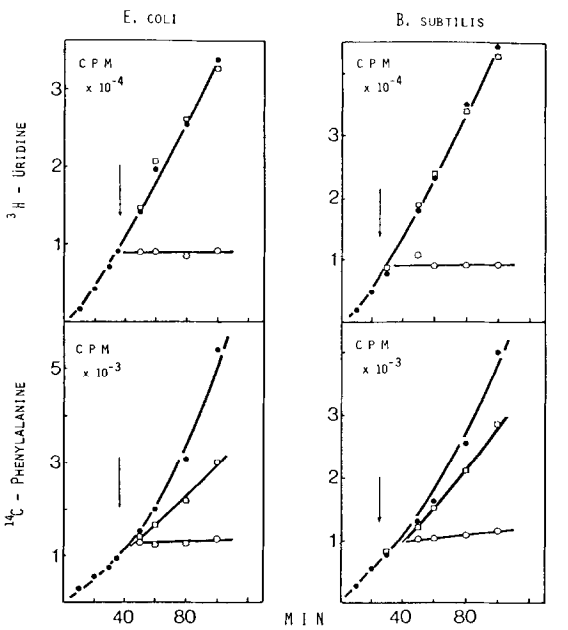

Figure 2. Effect of methionine on RNA and protein accumulation in *E. coli* or *B. subtilis* treated with trimethoprim.
Exponentially growing cultures were labeled with [^{3}H]uridine (5 μCi/ml; 62.5 Ci/mol) and [^{14}C]phenylalanine (0.25 μCi/ml; 2 Ci/mol). At the time indicated by the arrow trimethoprim (50 μg/ml) or trimethoprim plus methionine ($1x10^{-4}$M) was added. Samples (50 μl) were withdrawn and the incorporation of radioactivity into acid-insoluble material was determined as described.
● —— ● control; o —— o trimethoprim-treated; □ ——□ trimethoprim plus methionine.

TABLE I

Aminoacylation of tRNA and formylation of methionyl-tRNA in *E. coli* treated with trimethoprim; effect of methionine.

Growth condition	Percentage aminoacylation of				Percentage formylation of methionyl-tRNA
	$tRNA^{Met}$	$tRNA^{Gly}$	$tRNA^{Phe}$	$tRNA^{Leu}$	
Control	85	--	95	100	67
TRM	12	85	75	90	--
TRM plus methionine	75	100	100	100	2

One exponentially growing culture of *E. coli* MRE 600 (200 ml, A_{578} about 0.5) was used as control, the other was treated with trimethoprim (50 µg/ml) for 60 minutes, to the third culture trimethoprim was added and 20 minutes later methionine ($1x10^{-4}$M) for a further 40 minutes. Extraction of tRNA and the determination of aminoacylation and of formylation are described in the Materials and Methods section.

Determination of charged tRNA in vivo.

The fraction of tRNA aminoacylated in vivo was determined according to Folk and Berg (18) with the modifications of Lewis and Ames (19). Crude enzyme extracts were prepared according to Nirenberg (20) and used as a source of aminoacyl-tRNA synthetases.

Determination of methionyl-tRNA formylated in vivo.

Cells were labeled for 3 minutes with [35S]methionine (0.25 µCi/ml; 1 µCi/10 nmol). The tRNA was prepared under acidic conditions in order to conserve the ester bond between tRNA and amino acid (18). This procedure did not destroy formylmethionine. After deacylation of tRNA, methionine was separated from formylmethionine by cation exchange column chromatography. The quantitation of both labeled compounds was performed by scintillation counting (21). The method will be described and discussed elsewhere (22).

RESULTS AND DISCUSSION

Cultures of *E. coli* MRE 600 or *B. subtilis* W23 were grown in a glucose-salt medium supplemented with purines, thymidine and a mixture of all common amino acids except methionine. Upon treatment with trimethoprim (50 µg/ml) growth, RNA and protein synthesis stopped immediately. After the addition of methionine to the inhibited cultures the synthesis of RNA was completely

TABLE II

Aminoacylation of tRNA and formylation of methionyl-tRNA in *B. subtilis* treated with trimethoprim; effect of methionine.

Growth condition	Percentage aminoacylation of				Percentage formylation of methionyl-tRNA
	$tRNA^{Met}$	$tRNA^{Tyr}$	$tRNA^{Phe}$	$tRNA^{Leu}$	
Control	85	80	80	80	70
TRM	20	100	95	100	--
TRM plus methionine	90	100	85	100	2

Exponentially growing cultures of *B. subtilis* W23 (200 ml, A_{578} about 0.5) were treated as described for *E. coli* of table I.

restored whereas growth and protein synthesis remained partially restricted (Fig. 1 and 2). This indicates that under the defined conditions trimethoprim creates a starvation for methionine causing both the arrest of protein synthesis and, as a consequence of stringent control, the stop of RNA synthesis.

The signal for the stringent control mechanism is an uncharged tRNA at the ribosome (3,4,5). As shown in table I for *E. coli* MRE 600 and in table II for *B. subtilis* W23 the degree of aminoacylation of methionine-accepting tRNA was strongly decreased by treatment of the cells with trimethoprim. After the addition of methionine the level of methionyl-tRNA was restored to control values.

Guanosine tetraphosphate (ppGpp), the unusual nucleotide, that is assumed to mediate the stringent control mechanism (4,6,7,8), accumulated in *E. coli* or in *B. subtilis* during treatment with trimethoprim (Fig. 3). The addition of methionine abolished the accumulation of ppGpp.

In order to see as to whether the extent of formylation is correlated with the phenomenon of stringent control we have measured the intracellular content of formylated methionyl-$tRNA_f^{Met}$ (Table I and II). In

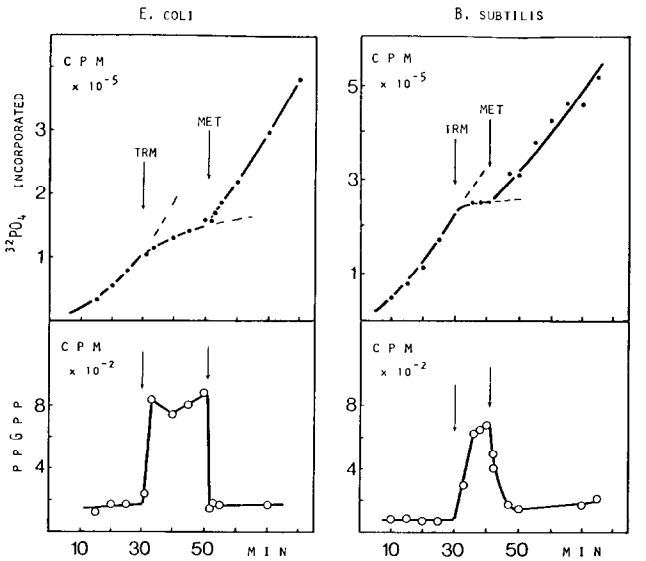

Figure 3. Accumulation of guanosine tetraphosphate (ppGpp) in *E. coli* or *B. subtilis* treated with trimethoprim and the effect of methionine. *E. coli* MRE 600 was grown in Tris-buffered glucose-salt medium containing phosphate at a concentration of 5×10^{-4}M. [^{32}P]orthophosphate was added at 200 μCi/ml. *B. subtilis* W23 was grown in the same medium containing [^{32}P]orthophosphate at 400 μCi/ml. Samples were subjected to trichloroacetic acid precipitation or formic acid extraction for ppGpp determination (6). The upper panels represent the incorporation of [^{32}P] into nucleic acids. The lower panels show the accumulation of ppGpp. Trimethoprim (50 μg/ml) and methionine (1×10^{-4}M) were added at time indicated by the arrows.

control cultures about two thirds of methionyl-tRNA have been found to be formylated. In trimethoprim-treated cells, during the starvation for methionine, the aminoacylation of methionine accepting tRNA was very low. The degree of formylation of the residual methionyl-$tRNA_f^{Met}$ has therefore not been determined. After the addition of methionine to the trimethoprim-treated cultures the degree of aminoacylation of $tRNA^{Met}$ had been restored to control values, the formylation, however, was only about two percent. Nevertheless the content of ppGpp had dropped down upon the addition of methionine (Fig. 3). The synthesis of RNA immediately resumed as was followed by the incorporation of [^{3}H]uridine or [^{32}P]orthophosphate into nucleic acids (Fig. 2 and 3). The effect of methionine on RNA synthesis

and ppGpp content in *E. coli* treated with trimethoprim has also been observed by Smith and Midgley (23,24).

We conclude that in the presence of trimethoprim the stringent response of RNA synthesis results solely from the inhibition of methionine synthesis. The unformylated initiator tRNA seems not to be a signal for the stringent control mechanism. Our results thus strongly argue against this assumption of other authors (10,14,15). Whether another step of initiation of protein synthesis may be involved in the stringent control mechanism remains to be examined.

ACKNOWLEDGEMENT

We are indebted to Drs. Helga and Walter Kersten for helpful discussions and critical reading of the manuscript. The studies were supported by the Deutsche Forschungsgemeinschaft Ke 98/11 and the Bundesministerium für Forschung und Technologie SN 6055.

REFERENCES

1. Sands, M. K., and Roberts, R. B. (1952) J. Bacteriol. 55, 505-511
2. Edlin, G., and Broda, P. (1968) Bacteriol. Rev. 32, 206-226
3. Fangman, W. L., and Neidhardt, F. C. (1964) J. Biol. Chem. 239, 1844-1847
4. Haseltine, W. A., and Block, R. (1973) Proc. Natl. Acad. Sci. U.S. 70, 1564-1568
5. Pedersen, F. S., Lund, E., and Kjeldgaard, N. O. (1973) Nature New Biol. 243, 13-15
6. Cashel, M., and Gallant, J. (1969) Nature 221, 838-841
7. Cashel, M. (1969) J. Biol. Chem. 244, 3133-3141
8. Fiil, N., von Meyenburg, K., and Friesen, J. D. (1972) J. Mol. Biol. 71, 769-783
9. Cashel, M. (1970) Cold Spring Harbor Symp. Quant. Biol. 35, 407-413
10. Lund, E., and Kjeldgaard, N. O. (1972) Eur. J. Biochem. 28, 316-326
11. Burchall, J. J., and Hitchings, G. M. (1965) Mol. Pharmacol. 1, 126-136
12. Blakeley, R. L. (1969) The biochemistry of folic acid and related pteridines. John Wiley and Sons, Inc., New York.
13. Marcker, K. (1965) J. Mol. Biol. 14, 63-70
14. Shih, A., Eisenstadt, J., and Lengyel, P. (1966) Proc. Natl. Acad. Sci. U.S. 56, 1599-1605
15. Khan, S. R., and Jamazaki, H. (1972) Biochem. Biophys. Res. Comm. 48, 169-174
16. Arnold, H. H., and Kersten, H. (1973) FEBS Letters 36, 34-38
17. Ogilvie, A., Lämmermann, M., Wiebauer, K., and Kersten, W. (1975) Biochim. Biophys. Acta 395, 136-145
18. Folk, W. R., and Berg, P. (1970) J. Bacteriol. 102, 204-212
19. Lewis, J. A., and Ames, B. N. (1972) J. Mol. Biol. 66, 131-142
20. Nirenberg, M. W. (1963) in Methods in Enzymology Vol. VI, p. 17-23 (Colowick, S. P., and Kaplan, N. O. eds.) Acad. Press, New York
21. Bray, G. A. (1960) Anal. Biochem. 1, 279-285
22. Arnold, H. H. submitted to Biochim. Biophys. Acta
23. Smith, R. J., and Midgley, J. E. M. (1973) Biochem. J. 136, 225-234
24. Smith, R. J., and Midgley, J. E. M. (1973) Biochem. J. 136, 249-257