ON THE CONTROL OF RNA SYNTHESIS IN STREPTOMYCIN-TREATED BACTERIA

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(Received for publication January 12, 1983)

Bacteria have evolved a number of regulatory mechanisms to maintain a balanced cellular composition in response to environmental variations. In particular, when the availability of any amino acid becomes limiting, protein synthesis is drastically diminished and, concomitantly, stable RNA synthesis is also strongly reduced[1]. Such interdependency of macromolecular synthesis is part of a rich array of metabolic adjustments grouped together under the general term of "stringent response"[2,3]. It is associated with the accumulation of unusual nucleotides within the cells, namely guanosine tetraphosphate ppGpp, and its physiological precursor, guanosine pentaphosphate pppGpp[4]. Since an inverse correlation between ppGpp concentration and the rate of stable RNA synthesis has been almost invariably observed under a great variety of conditions of amino acid deficiency, this nucleotide has been proposed to play a role in the stringent response[5].

The stringent control of RNA synthesis during amino acid limitation is specific to stringent strains of *relA*$^+$ genotype, and can be abolished by single-site mutations which confer a "relaxed" phenotype to bacteria[4,5]. Thus, relaxed mutants (*relA*) keep synthesizing stable RNA during starvation and do not accumulate guanosine polyphosphates.

Another situation has been described which also leads to the uncoupling of protein and RNA syntheses. When stringent cells are treated with certain antibiotics such as chloramphenicol, sparsomycin or erythromycin, protein synthesis is blocked but RNA synthesis proceeds at a near-normal rate[6,7]. Similarly, when bacteria are first subjected to amino acid starvation then treated with the same drugs, protein synthesis remains arrested but RNA formation quickly resumes. Although the precise mechanism of such resumption has still to be determined, it is known to be accompanied by a rapid decrease of the intracellular level of ppGpp[6,7]. The above antibiotics share in common the property of interacting with the large 50S ribosomal subunit of bacteria[8], that is the same subunit which harbors the stringent factor, *i.e.*, the *relA* gene product, responsible for the formation of ppGpp[9]. In view of these observations, it seemed of interest to analyze in comparison the effects on RNA synthesis and ppGpp accumulation of an antibiotic, the aminoglycoside streptomycin, which interacts specifically with the small 30S subunit of ribosomes[8,10].

In this study, the otherwise isogenic pair of *Escherichia coli* strains CP78 (*relA*$^+$) and CP79 (*relA*) was used[11]. Both strains require arginine, leucine, threonine and histidine for growth. Cells were cultured at 37°C in a tris-glucose medium[12] supplemented with the four essential amino acids (50 μg/ml each) and containing 1 mM KH_2PO_4. Starvation experiments were performed by transferring exponentially growing cells to a fresh medium lacking arginine. The synthesis of protein and RNA was measured by the incorporation of, respectively, [^{14}C]proline (0.04 μCi and 10 μg/ml) and [^{3}H]uracil (0.15 μCi and 5 μg/ml) into cold 5% trichloroacetic acid-precipitates. The radioactivity was counted in a scintillation fluid using the appropriate double-label setting in a Tri-Carb Packard spectrometer. For the determination of nucleotide concentrations, the method previously described[13] was used. Briefly, cells were labeled with [^{32}P]orthophosphate (170~200 μCi/ml), 1 ml aliquots were withdrawn and treated with an equal volume of 2 M formic acid for 30 minutes at 0°C. The suspension was then subjected to low-speed centrifugation, and 10 to 20 μl of formic acid extract was analyzed by ascending chromatography on polyethyleneimine cellulose plates. The nucleotides were localized by autoradiography, and their concentration was calculated and expressed as picomol per ml of culture medium. Streptomycin sulfate was obtained from Boehringer Mannheim Co. and kasugamycin sulfate from Serva Feinbiochemica. Radioactive compounds were from the French C.E.A. All other reagents were of analytical grade from Sigma Chemical Co. or Merck Co.

The effects of amino acid starvation on the syn-

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Fig. 1. Protein and RNA synthesis in *E. coli* cells under amino acid starvation and/or streptomycin treatment.

Cells were initially grown in exponential phase in the presence of radioactive proline and uracil then subjected to various treatments (arrow), and the amount of radioactivity incorporated into the acid-insoluble material was determined as a function of time.

Diagrams a, a', b and b': arginine starvation (■) or streptomycin action (□: 200 μg/ml; ▲: 600 μg/ml). Diagrams c and c': both arginine starvation and 200 μg/ml (□) or 600 μg/ml (▲) streptomycin. Diagrams d and d': arginine starvation (■) followed 20 minutes later by treatment with 200 μg/ml (□) or 600 μg/ml (▲) streptomycin. In all cases, untreated cells were analyzed as a control (●).

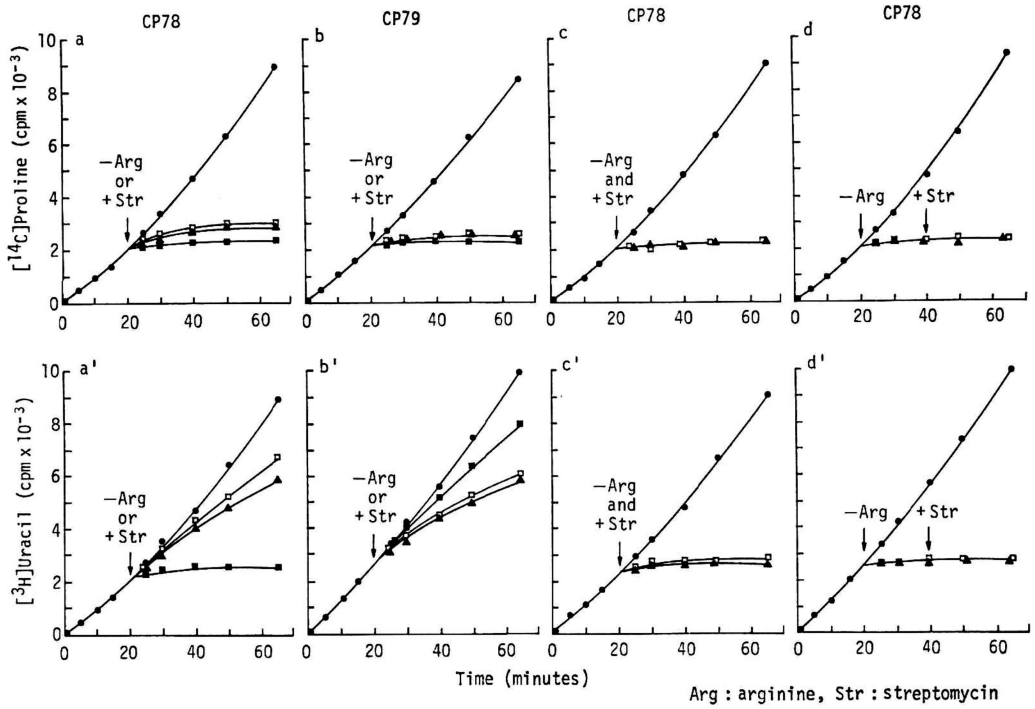

Table 1. Effects of amino acid starvation and/or aminoglycoside treatment on ppGpp and pppGpp levels in *E. coli* cells.

Treatment	ppGpp (pmol/ml)			pppGpp (pmol/ml)		
	5 minutes	20 minutes	40 minutes	5 minutes	20 minutes	40 minutes
Starvation	152	128	123	44	31	34
Streptomycin (200 μg/ml)	0	0	0	0	0	0
Streptomycin (600 μg/ml)	0	0	0	0	0	0
Kasugamycin (600 μg/ml)	0	0	0	0	0	0
Starvation+streptomycin (200 μg/ml)	156	113	82	33	26	12
Starvation+streptomycin (600 μg/ml)	151	89	50	38	19	9
Starvation+kasugamycin (600 μg/ml)	0	0	0	0	0	0

thesis of protein and RNA, and on the formation of guanosine polyphosphates, were first analyzed in a control experiment. The data presented in Fig. 1 show that arginine deprivation results in a drastic inhibition of protein synthesis in both stringent and relaxed strains (diagrams a and b). Simultaneously, RNA synthesis is strongly reduced in the former (diagram a') whereas it is only slightly affected in the latter (diagram b') as expected[14]. Also, ppGpp and pppGpp accumulate in stringent cells during the first 5 minutes of starvation, reaching a steady-state value which is maintained for at least 35 minutes (Table 1). No such accumulation of these nucleotides oc-

curs in the relaxed mutant.

When analyzing the action of streptomycin, a similar blockade of protein synthesis is observed, in either type of strain, under treatment with 200 or 600 μg/ml of drug (diagrams a and b). Such high concentrations of antibiotic were necessary in order to induce complete inhibition of protein synthesis as during amino acid starvation, which allows a direct comparison of the two situations. At these doses, RNA synthesis still proceeds at a significant rate in stringent as well as in relaxed cells (diagrams a′ and b′). It thus appears that, as far as RNA production is concerned, the effect of streptomycin in stringent cells is quite different from that of amino acid starvation. Consistent with this finding, it has previously been reported[15] that streptomycin stimulates RNA synthesis in bacteria even when used at relatively low doses ranging from 30 to 80 μg/ml. We have determined that no guanosine polyphosphate accumulates during drug treatment (Table 1), which supports the concept that ppGpp plays a key role in the regulation of RNA synthesis[2,5].

A similar situation was encountered when measuring, in a parallel study, the effects of kasugamycin, another aminoglycoside inhibitor interacting with 30S ribosomal subunits[8]. Indeed, no guanosine polyphosphate accumulation was observed under treatment of cells with 600 μg/ml of this drug (Table 1) which blocked almost completely protein synthesis without affecting substantially RNA formation (not shown), as with streptomycin.

In another set of experiments, macromolecular syntheses were measured in stringent cells subjected simultaneously to both arginine starvation and streptomycin treatment. The results displayed in Fig. 1 (diagrams c and c′) show that, in this case, significant production of RNA does not occur while protein synthesis is arrested, which suggests that the stimulatory effect of streptomycin (as described above) is shut off by the lack of an amino acid essential to growth. Under these conditions, ppGpp and pppGpp accumulate within the cells (Table 1), but their respective concentrations, similar to those measured under simple starvation during the first 5 minutes, decrease progressively as a function of time. After 40 minutes in the presence of 600 μg/ml of antibiotic, the concentration of ppGpp is less than half its value in arginine-starved cells (50 *vs.* 123 pmol/ml) and the concentration of pppGpp is reduced by a factor of about 4 (9 *vs.* 34 pmol/ml). It therefore seems that streptomycin restricts the formation of guanosine polyphosphates, which is in agreement with the earlier finding that the nucleotides are not produced by bacteria treated with the drug alone. Nevertheless, this restriction is not extensive enough to reverse the blockade of RNA synthesis by lowering the pools of nucleotides below the threshold level required for exerting their inhibitory action. This interpretation is favored by the last experiment reported here (Fig. 1, diagrams d and d′). When streptomycin (200 or 600 μg/ml) is added to bacteria previously deprived of arginine for 20 minutes, no significant resumption of RNA synthesis is observed during the 25 minutes following drug addition. Moreover, it is worth noting that, contrary to the observation made with streptomycin, neither ppGpp nor pppGpp are found to accumulate when starving bacteria are treated with kasugamycin (Table 1).

In conclusion, quantitative, if not qualitative, differences emerge when comparing streptomycin to other antibiotics, *e.g.*, chloramphenicol, sparsomycin, erythromycin[6,7] or kasugamycin (our present results), with regard to their capacity to interfere with the stringent control system, namely by reversing the restriction of RNA synthesis triggered by amino acid starvation. This differential effect of antibiotics could be related to their mode of interaction with ribosomes. Thus, for example, the ribosomal protein mainly involved in the binding of chloramphenicol to ribosomes is protein L_{16} from the 50S subunit (for review, see reference 8). Similarly, proteins L_{15} and L_{16} are crucially required for the binding of erythromycin to 50S particles. These same proteins are involved in the stimulation of the synthesis of ppGpp catalyzed by stringent factor[16]. Consequently, it is conceivable that such antibiotics affect the ribosome-dependent production of ppGpp by interacting with proteins of the 50S ribosome that are necessary for stringent factor activity. By contrast, streptomycin interacts with the 30S subunit and, moreover, most of the ribosomal proteins involved in its binding such as proteins S_8, S_5, S_{10}, S_{14} and S_{19}[8,10], have only a small stimulatory effect on the stringent factor activity[16]. Therefore, it can be envisaged that streptomycin would not affect primarily the functioning of the stringent factor, which could explain its delayed action on ppGpp synthesis.

Further data are however needed to check the plausibility of this hypothesis, especially for those antibiotics whose mode of interaction with ribosomes is still not precisely known at the molecular level. In the case of kasugamycin, it is possible that this drug interacts with part or all of ribosomal proteins from the 30S subunit (S_4, S_7, S_9 and S_{11}) required for stringent factor activity[16] and, consequently, interferes with the formation of ppGpp.

The present results can also be discussed in terms of translational accuracy; it has previously been suggested[17] that ppGpp is responsible for the maintenance of qualitatively correct translation when protein synthesis is reduced by amino acid starvation. On the other hand, it has been shown that streptomycin causes mistranslation in bacteria while kasugamycin does not[8]. Our data now indicate that ppGpp does not accumulate in the presence of either streptomycin or kasugamycin alone. It can therefore be concluded that no permanent relationship seems to exist between the intracellular level of ppGpp and the degree of misreading during restriction of protein synthesis. The proposed role of this nucleotide as a regulator of translational fidelity during amino acid shortage[17] does not seem to apply to bacteria under aminoglycoside antibiotic inhibition.

Acknowledgments

This work was supported by the Centre National de la Recherche Scientifique (ERA 399) and the Mission de la Recherche.

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