BREAKDOWN OF ppGpp IN *spoT*$^+$ AND *spoT*$^-$ CELLS OF *ESCHERICHIA COLI*

Manganese and energy requirement and tetracycline inhibition

Herman A. DE BOER, Albertus J. BAKKER and Max GRUBER
Biochemisch Laboratorium, The University, Zernikelaan, Groningen, The Netherlands

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1. Introduction

The reaction pathway and the mechanism of degradation of the regulating nucleotide guanosine 3′-diphosphate, 5′-diphosphate (ppGpp), are unknown. The intracellular ppGpp concentration is controlled by regulation of the rate of its synthesis [1–3] and the rate of its breakdown [4–7]. Elucidation of the mechanism of ppGpp synthesis [2,8] has led to the understanding of its control mechanism. Obviously, the same will hold for ppGpp breakdown.

In vivo, ppGpp breakdown is a rapid process. First-order degradation rate constants up to 3.0 min^{-1} have been found [1–15]. However, ppGpp is not broken down in permeabilised cells [16], a preparation of membrane vesicles [17], in vitro systems for synthesis of ribosomal RNA [18], or ppGpp or proteins and in crude cell extracts (unpublished observations). Only in the cold-shocked cell preparations of Raué and Cashel [11], previously synthesized intracellular ppGpp is broken down. Unfortunately, added ppGpp did not penetrate the remaining cell-wall barrier [11]. Obviously, the in vitro systems lack necessary components which have been lost or inactivated during preparation. Their nature may become clearer by studying ppGpp breakdown in vivo.

As has been shown several times [4–7] the rate of ppGpp breakdown is related to the rate of high-energy phosphate bond production. Mutants (*spoT*$^-$) which are impaired in ppGpp breakdown (k_{degr} = 0.1 min^{-1}) have been described [9–15,19]. Hence, the *spoT* gene product is probably an enzyme or a component of an enzyme [20], catalysing ppGpp breakdown [9–15,19].

Several reports [10,11] have appeared which show that in *spoT*$^-$, in contrast to *spoT*$^+$ cells, ppGpp breakdown is blocked by the addition of tetracycline (0.5 mM). In cold-shocked *spoT*$^-$ cells manganese ions appeared to be essential for ppGpp breakdown; again, this was not found for cold-shocked *spoT*$^+$ cells. These data have been interpreted [10,11] as indicating two pathways for ppGpp breakdown the major of which was assumed to be eliminated in *spoT*$^-$ strains. On the other hand, Raué and Gruber [12] proposed a single pathway for ppGpp breakdown with a changed enzyme. In the present paper we show that in *spoT*$^+$ cells tetracycline also inhibits ppGpp breakdown, albeit at higher concentrations, and also show a manganese requirement in *spoT*$^+$ strains. We demonstrate that the tetracycline sensitivity in *spoT*$^+$ strains can be increased by energy deprivation. We conclude from our results to a single pathway for ppGpp breakdown in both genotypes. We suggest that the *spoT* mutation is in the structural gene and leads to a mutant enzyme with a changed manganese binding and, as a consequence, increased tetracycline sensitivity.

2. Materials and methods

2.1. *Bacterial strains and culture conditions*

E. coli W 1 (*leu*$^-$, *arg*$^-$, *his*$^-$, *thr*$^-$, *pro*$^-$, *thi*$^-$, *rel*$^+$, *spoT*$^+$) and *E. coli* W 2, isogenic with W 1 except the *spoT* locus were used. They were kindly provided by Dr J. Gallant who constructed them. The bacteria were exponentially growing in a Tris/glucose low-phosphate medium with the appropriate supplements and always labeled with ^{32}P one generation time before

starting the experiment. For further details see legends and ref. [3,7,13].

2.2. *Methods*

For determination of nucleotide pools and rate constants for ppGpp breakdown and materials used see ref. [3,7,13]. Tetracycline was obtained from Sigma Chemical Co.

3. Results and discussion

3.1. *Inhibition of ppGpp degradation by tetracycline in amino acid starved cells of* E. coli *W 1* (spoT$^+$) *and* E. coli *W 2* (spoT$^-$)

Stamminger and Lazzarini [10], as well as Raué and Cashel [11] showed that tetracycline in a concentration of 250 μg/ml (0.5 mM) in the medium blocks ppGpp breakdown in *spoT*$^-$ cells. The experiment shown in fig.1 confirms their results. Figure 1 also shows that much lower concentrations, even 10 μg/ml (20 μM), are already inhibitory (see also ref. [10]). In *spoT*$^+$ cells ppGpp breakdown is barely affected by 0.5 mM tetracycline. Higher concentrations of the drug, however, are inhibitory with a complete block occurring at 2.0 mM. Thus, both strains are susceptible to tetracycline; the difference only lies in the degree of sensitivity of the ppGpp degrading enzyme(s) to the drug. In both strains the degree of inhibition increases with the duration of the treatment. Apparently, the active transport [21–23] of the drug into the cell takes some time and inhibition or blocking is only visible after build-up of the intracellular tetracycline concentration. In the *spoT*$^+$ cells the conversion of pppGpp to ppGpp was not affected even at the highest tetracycline concentration mentioned (data not shown).

3.2. *Is manganese a cofactor of the ppGpp degrading enzyme(s)?*

Raué and Cashel [11] showed that ppGpp breakdown in cold-shocked *spoT*$^-$ cells required Mn^{2+}. Therefore, the inhibition of ppGpp breakdown by tetracycline might be assumed to be caused by chelation of manganese in both genotypes. Tetracycline concentrations which are as high as those used here inhibit several processes [24]. A number of (side) effects of tetracycline has been ascribed to the chelation of divalent cations. Tetracycline has a moderate affinity for Mg^{2+} and Mn^{2+} (K_a $10^{4.9}$ and K_a $10^{4.4}$,

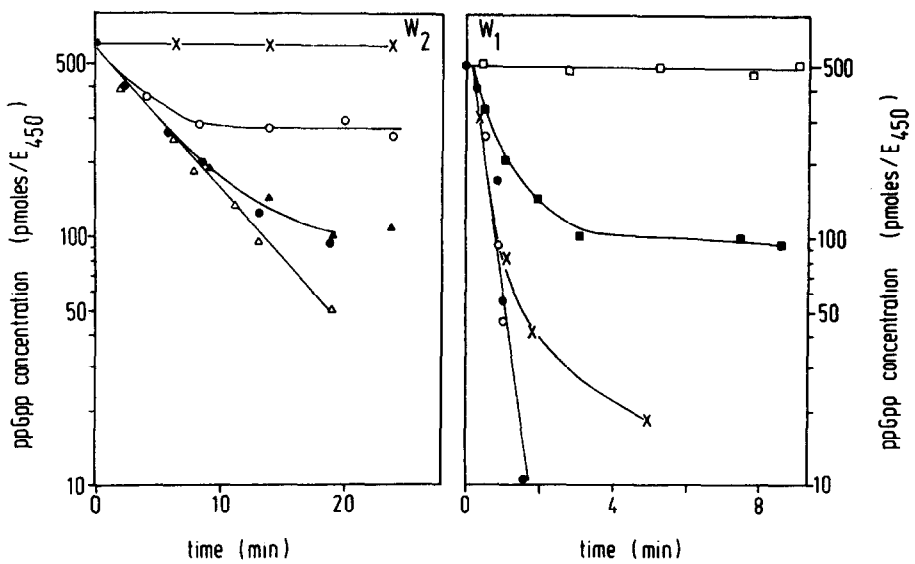

Fig.1. Effect of different tetracycline concentrations on ppGpp breakdown in *E. coli*. To prelabeled and exponentially growing cells of *E. coli* W 1 (*spoT*$^+$) and W 2 (*spoT*$^-$) at a density of $A_{450}^{1\,cm}$ 0.3, L-valine was added to a final concentration of 400 μg/ml. Ten minutes later different tetracycline concentrations were added to subcultures: (●——●) 10 μg/ml (20 μM); (▲——▲) 20 μg/ml; (○——○) 80 μg/ml; (X——X) 250 μg/ml (0.5 mM); (■——■) 500 μg/ml; (□——□) 1000 μg/ml (2 mM); (△——△) 100 μg/ml chloramphenicol. Left panel, W 2 (*spoT*$^-$). Right panel, W 1 (*spoT*$^+$). Note the different time scales.

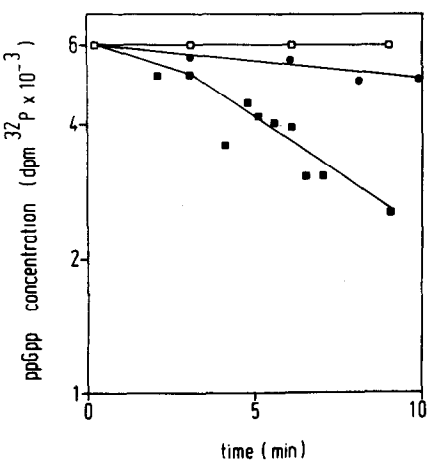

Fig.2. Effect of Mn^{2+} and Mg^{2+} on ppGpp breakdown in tetracycline treated cells of *E. coli* W 1 (*spoT*$^+$). At t −15 min, L-valine was added to prelabeled and exponentially growing cells. At t −5 min, tetracycline was added to a final concentration of 2.0 mM. At t 0 min to one part of the culture $MgCl_2$ (a 100-times concentrated solution in minimal medium) was added to a final concentration of 4.0 mM. To another part of the culture $MnCl_2$ was added to 4.0 mM final concentration. (□——□) Tetracycline alone. (●——●) tetracycline plus Mg^{2+}. (■——■) Tetracycline plus Mn^{2+}.

respectively; ref. [23–25]) and some, but not all, of its effects are counteracted by these ions [24].

As a first test of the assumption we studied the effect of Mg^{2+} and Mn^{2+} addition on the tetracycline inhibition of ppGpp breakdown in isoleucine-starved *spoT*$^+$ cells. We found that prior addition of 2.0 mM manganese completely protects ppGpp breakdown against 2.0 mM tetracycline. Magnesium has only a minor effect unless a small amount (0.1 mM) of manganese is added (data not shown). Figure 2 shows that manganese partly restores ppGpp breakdown in isoleucine-starved and tetracycline (2.0 mM) treated *spoT*$^+$ cells, whereas magnesium has only a slight effect.

Altogether, these results indicate that manganese is a cofactor in the reaction for ppGpp breakdown genotypes. The increased tetracycline sensitivity of this reaction in *spoT*$^-$ cells suggests that the binding of Mn^{2+} to the *spoT* gene product is weakened in the *spoT*$^-$ mutants.

3.3. *Inhibition of ppGpp breakdown in glucose-starved* spoT$^+$ *cells by tetracycline*

At inhibition of the high-energy phosphate-bond production, e.g., glucose starvation [7], carbon source shift-down [4], or treatment with 2,4-dinitrophenol [7] the rate constant for ppGpp breakdown in *spoT*$^+$ cells is lowered by an order of magnitude and thus approximates that measured in growing, or amino acid-starved, *spoT*$^-$ cells [9–15,19]. What is the mechanism of this phenomenon?

Formerly, the first step of ppGpp breakdown was assumed to be its phosphorylation to pppGpp [9–12]. Recently, this assumption has been rejected and pppGpp has been demonstrated to be the precursor, rather than the product of ppGpp [3,13–15,19]. Therefore, it now appears unlikely that a phosphate transfer to ppGpp plays a role in its degradation. More probably the reduction of the rate of high-energy phosphate bond production as such has a lowering effect on the activity of the ppGpp degrading enzyme(s). In this respect glucose-starved *spoT*$^+$ cells phenotypically resemble the *spoT*$^-$ genotype. For this reason we added tetracycline (0.5 mM) to glucose-starved cells and found that the tetracycline sensitivity was increased strongly (fig.3). The sensitivity to tetracycline could be restored to the original value when glucose starvation was relieved (and isoleucine starvation was evoked to elevate the ppGpp, and pppGpp, level). It should be noted that this increased sensitivity to tetracycline cannot be explained in terms of increased uptake of the drug. On the contrary, glucose starvation lowers the net uptake of tetracycline [21,22]. Thus, the actual difference in tetracycline sensitivity may be even greater. We conclude that the resemblance of glucose-starved *spoT*$^+$ cells and growing or isoleucine-starved *spoT*$^-$ cells also concerns their sensitivity to tetracycline. Therefore, the sensitivity of the ppGpp degrading enzyme(s) to tetracycline is determined not only by a genetic factor, i.e., the *spoT* gene, but also by a physiological factor. In view of the role of manganese in ppGpp breakdown we propose that glucose starvation as well as mutation (independently?) weaken the binding of manganese to the *spoT* gene product. An experiment combining glucose starvation and tetracycline inhibition in the *spoT*$^-$ cells is not feasible since glucose starvation alone already lowers the breakdown rate to the detection limit (see below).

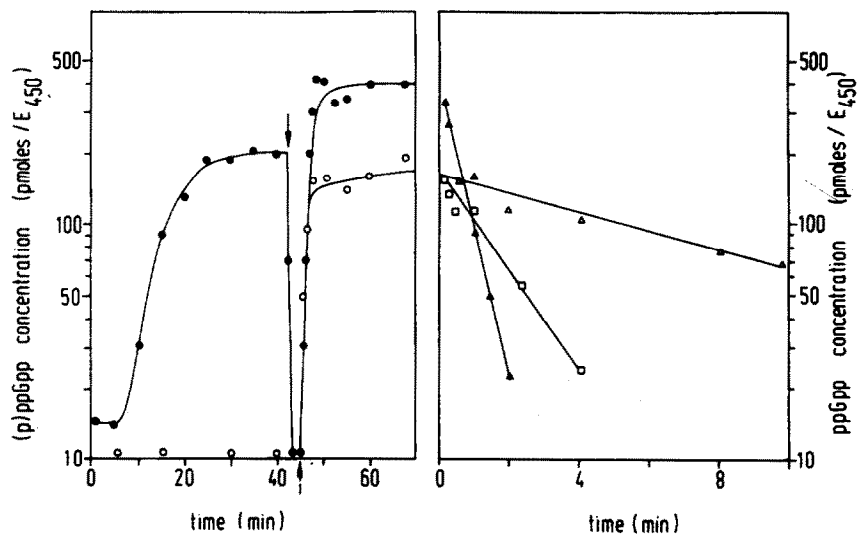

Fig.3. Effect of glucose starvation on the tetracycline sensitivity of the reaction for ppGpp breakdown in *E. coli* W 1 (*spoT*$^+$). Cells were grown in a minimal medium containing a limiting amount (0.024%) of glucose (see ref. [7]). At a cell density of $A^{1\,cm}_{450\,nm}$ 0.5 growth ceased; ppGpp accumulation started at *t* 5 min, due to reduction of the rate of its breakdown [7]. Left panel: (●——●) ppGpp; (○——○) pppGpp. At *t* 42 min, glucose starvation was relieved by the addition of glucose (0.4% final concentration) and consequently ppGpp disappeared quickly (k = 3.0 min^{-1}). In order to elevate the ppGpp concentration its synthesis was triggered (and hence that of pppGpp, ref. [3]) at 45 min by the addition of L-valine (200 μg/ml final concentration). As well during glucose starvation as after addition of glucose and L-valine the decay of ppGpp was followed in subcultures after the addition of two different tetracycline concentrations. The decay curves are shown on the right panel. (□——□) 20 μM (10 μg/ml) tetracycline or 100 μg/ml chloramphenicol, added at *t* 25 min to the glucose-starved culture (k = 0.44 min^{-1}). (△——△) 0.5 mM (250 μg/ml) tetracycline, added at *t* 25 min to the glucose-starved culture (k = 0.06 min^{-1}). (▲——▲) 0.5 mM tetracycline, added at *t* 55 min to which glucose and valine were added (k = 1.5 min^{-1}). Addition of 10 μg/ml tetracycline or 100 μg/ml chloramphenicol to the latter culture revealed a degradation rate constant of 2.6 min^{-1} (curves not shown; see also ref. [3]).

3.4. *Degradation of ppGpp in* spoT$^-$ *cells too is an energy-requiring process*

Table 1 shows that treatment with 2,4-dinitrophenol or glucose exhaustion applied to *spoT*$^-$ cells decreases the rate constant for ppGpp breakdown from 0.1 min^{-1} to about 0.02 min^{-1}. This is also evident from the study of Gallant et al. [4] showing energy-dependence of ppGpp breakdown for the first time. Their study was done with a strain which appeared later [9,10] to be a *spoT*$^-$. Thus, also with respect to the energy requirement of the reaction for ppGpp breakdown *spoT*$^+$ and *spoT*$^-$ strains do not differ. One might suggest that the degrading enzyme requires ppGpp–Mn instead of ppGpp as substrate. Removal of Mn^{2+} by tetracycline might lower the concentration of this complex. This suggestion, however, does not explain the difference between *spoT*$^+$ and *spoT*$^-$ cells unless one assumes that *spoT*$^-$ cells accumulate tetracycline to a higher concentration than *spoT*$^+$ cells. This suggestion can also not explain the increased tetracycline sensitivity of glucose-starved *spoT*$^+$ cells. In such cells tetracycline accumulation is impaired [21,22].

It is also unlikely that mutation of the *spoT* gene only results in a lowering of the concentration of the ppGpp degrading enzyme since in that case its tetracycline sensitivity would not be altered. The simplest explanation of the difference between *spoT*$^+$ and *spoT*$^-$ is thus a mutation in the structural part of the *spoT* gene resulting in a weakened binding of Mn^{2+} by the enzyme catalyzing ppGpp breakdown, which in turn results in a greater sensitivity towards tetra-

Table 1
First order rate constants for ppGpp breakdown in glucose-starved and in 2,4-dinitrophenol-treated cells

Strain	Treatment		
	Valine	Valine + DNP	Glucose starvation
	(degradation rate constants, k min^{-1})		
W 1 (*spoT*$^+$)	3.0	0.1	0.3
W 2 (*spoT*$^-$)	0.1	0.02	0.02

At t 0 min isoleucine starvation was evoked. Two minutes later 2,4-dinitrophenol (DNP) was added (2.0 mM final concentration). Glucose starvation in another culture was evoked by growing the cells on a limiting amount of glucose (0.024%). Five minutes after DNP treatment or glucose exhaustion the new rate constant for ppGpp breakdown was established (see ref. [7]). These values are given.

cycline. Energy deprivation, at least in the wild type (*spoT*$^+$) has a similar effect on Mn^{2+} binding. We thus propose that the ppGpp degradation system is the same in *spoT*$^+$ and *spoT*$^-$ strains since they are subject to the same conditions and effectors. The nature of the pathway for ppGpp breakdown and the effect of energy deprivation upon it will probably only be elucidated when ppGpp degradation can be studied in a cell-free system. Our results point to some conditions necessary for the preparation of such a system.

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