ALTERED METABOLISM OF ppGpp IN QUINONE-TREATED *E. COLI*: POSSIBLE ROLE OF AMINOACYL-tRNA SYNTHETASES

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Received March 5,1976

SUMMARY: The bacteriostatic quinone 6-amino-7-chloro-5,8-dioxoquinoline inhibits leucyl-tRNA synthetase in vivo and in vitro (Ogilvie et al. Biochim. Biophys. Acta 407, 357-364; 1975). In this report it is shown that the quinone also interferes with the metabolism of ppGpp. Quinone treatment of *E. coli* MRE 600 causes the same phenotypic pattern as found in spoT$^-$ mutants: overproduction of ppGpp and a drastic increase of its half-life; the formation of pppGpp, the possible degradation product of ppGpp, is blocked. A model is discussed to explain how the inhibition of leucyl-tRNA synthetase could account for the altered metabolism of ppGpp.

INTRODUCTION: When *Escherichia coli* cells are deprived of an essential amino acid the synthesis of stable RNA is immediately curtailed. During this "stringent response" the unusual nucleotides guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) are largely accumulated (1,2). The putative role of ppGpp as the negative mediator of the stringent control system is mainly based on a strict kinetic and quantitative correlation of its increase with the inhibition of RNA synthesis (3). Furthermore ppGpp was found to inhibit RNA polymerase in vitro (4). Guanosine pentaphosphate is not necessary for the stringent control system but seems to be a product of degradation of ppGpp. These conclusions are drawn from the characteristics of a spoT$^-$ mutant of *E. coli*: (i) the "spotless" mutant is unable to accumulate pppGpp but shows stringent control, (ii) ppGpp is overproduced and (iii) its half-life has markedly increased (5,6). It has been proposed that ppGpp is converted to pppGpp by the spoT gene product, although the precise mechanism is unclear.

ABBREVIATIONS: ppGpp, guanosine 3'-diphosphate-5'-diphosphate; pppGpp, guanosine 3'-diphosphate-5'-triphosphate; ApppGpp, guanosine 3'-diphosphate-5'-adenosine-5'-(p^1,p^3-triphosphate); HEPES, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid.

The results obtained from wild type cells treated with 6-amino-7-chloro-5,8-dioxoquinoline substantiate the catabolism of ppGpp via pppGpp as deduced from experiments with the spoT$^-$ mutant.

The function of aminoacyl-tRNA synthetases in the metabolism of ppGpp has been postulated (7). The observation that the quinone tested causes stringent response by inhibiting leucyl-tRNA synthetase (8,9) has led me to discuss how this interaction could account for the altered metabolism of guanosine tetraphosphate in quinone-treated cells.

MATERIALS AND METHODS

Growth conditions. *Escherichia coli* MRE 600 was used throughout this investigation. The cells were grown in Tris-buffered glucose-salt medium (8). The concentration of phosphate was 5×10^{-4} M. All experiments were carried out with exponentially growing cells as monitored photometrically at 578 nm.

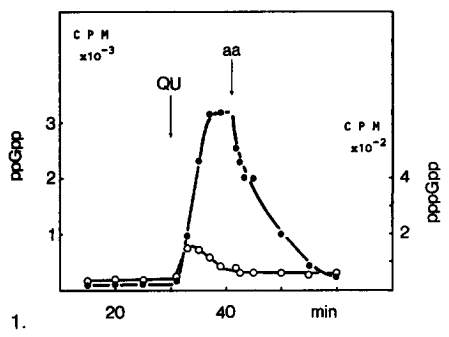
Determination of ppGpp and pppGpp concentrations. The incorporation of [^{32}P]-orthophosphate into the unusual guanosine nucleotides was measured as described by Cashel (2).

Chemicals. Alpha-hydrazino caproic acid (α-HCA) was a gift from Dr. R. Cortese, Naples. The amino acid analogue was used to impose amino acid starvation in *E. coli* MRE 600. Its inhibitory effect on growth can immediately be reversed by the addition of a mixture of all common amino acids, methionine being essential for the relief. The quinone 6-amino-7-chloro-5,8-dioxoquinoline was a gift from Dr. Pütter, Farbwerke Bayer, Wuppertal, Germany.

Leucyl-tRNA synthetase (EC 6.1.1.4) from *E. coli* MRE 600 was partially purified by ammonium sulfate precipitation and DEAE-cellulose chromatography as described (10); 2-mercaptoethanol was removed by dialysis. Assay conditions are given in the legend of figure 4.

RESULTS AND DISCUSSION

The quinone 6-amino-7-chloro-5,8-dioxoquinoline inhibits growth of *E. coli* by interfering with leucyl-tRNA synthetase (8,9). This interference leads to an increase of uncharged tRNA^{Leu} in the treated cells, thus creating the condition of the stringent control mechanism (11,12,13). There is a drastic increase of guanosine tetraphosphate (ppGpp) in quinone-treated cells whereas guanosine pentaphosphate (pppGpp) accumulates only for some minutes to a relatively small level (Fig. 1). After the addition of amino acids



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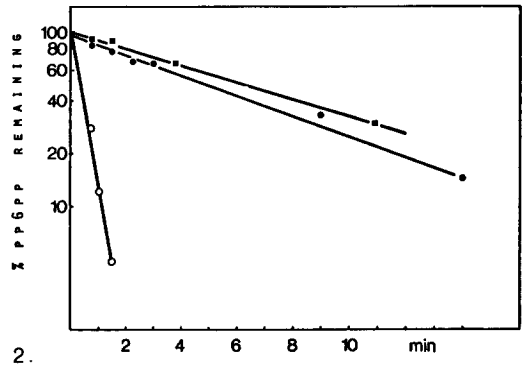

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Figure 1
Accumulation of ppGpp (● —— ●) and pppGpp (o —— o) in *E.coli* MRE 600 treated with 6-amino-7-chloro-5,8-dioxoquinoline (QU); effect of amino acids. An exponentially growing culture at an optical density of 0.200 (A_{578}) was labeled with [^{32}P] orthophosphate (80 μCi/ml) for 30 min before quinone (QU) was added to a final concentration of 2 μg/ml. About 11 minutes later a mixture of amino acids was added (aa; 6×10^{-5} each). The mixture contained leucine in combination with all common amino acids to avoid secondary starvation effects (9); cysteine, not necessary for the relief of inhibition, was only omitted to prevent reaction with the quinone.
To determine the relative amounts of ppGpp and pppGpp aliquots (50 μl) were withdrawn and quickly mixed with ice-cold formic acid (50 μl; 2 M). After centrifugation 20 μl of the acidic extract were chromatographed on polyethyleneimine-cellulose-coated glass plates with 1.3 M KH_2PO_4 (pH 3.4). Detection by autoradiography and counting procedures of the spots were performed as described by Cashel (2).

Figure 2
The kinetics of ppGpp decay in amino acid starved *E. coli* MRE 600 and in cells treated with 6-amino-7-chloro-5,8-dioxoquinoline.
Exponentially growing cells, labeled with [^{32}P] orthophosphate (80 μCi/ml) were treated with α-hydrazino caproic acid (4×10^{-4} M) for 15 minutes, reaching the steady state level of ppGpp. A mixture of amino acids (see legend of fig. 1) was added, and at the times indicated (O——O) aliquots were withdrawn to determine the amounts of ppGpp. The values were plotted as per cent of the steady state level versus time.
Two parallel cultures were pretreated with quinone (●——● 2 μg/ml; ■——■ 6 μg/ml) instead of α-hydrazino caproic acid and then treated with amino acids.

ppGpp disappears. The kinetic analysis of the degradation of ppGpp revealed a first order reaction with a rate constant of 0.14 min^{-1} (Fig. 2). For comparison the catabolism of ppGpp has been investigated during the relief of amino acid starvation induced by α-hydrazino caproic acid (Fig. 2). The decay rate has been found to be about 2 min^{-1}, which is in accordance with published values for amino acid starved cells (2,5). Thus in quinone-treated

(2 µg/ml) cells the catabolic capacity for ppGpp seems to be more than ten times lower. Three times higher amounts of quinone (6 µg/ml) did not further diminish significantly this capacity (Fig. 2). A similar residual rate of degradation has also been found in spoT$^-$ cells (5,6).

The steady state level of ppGpp in amino acid starved cells is assumed to be the resultant of the maximal rate of synthesis and a normal rate of degradation (2,14,15). The quinone provides the condition of amino acid starvation (8,9) and obviously inhibits the degradation of ppGpp. Therefore it has been expected that the quinone, added to amino acid starved cells, would further elevate the level of ppGpp; this has actually been observed (Fig. 3). The quinone was added when ppGpp and pppGpp had reached their steady state levels in cells treated with α-hydrazino caproic acid. Simultaneously with the increase of ppGpp, the level of the pentaphosphate was diminished (Fig. 3). After a lag of about 40 seconds the rapid decay of pppGpp followed a first order kinetic (rate constant 0.9 min^{-1}), indicating that the synthesis of guanosine pentaphosphate had been blocked by the quinone.

From the characteristics of the spoT$^-$ mutant it has been concluded that ppGpp is converted to pppGpp (5,6). The putative phosphorylating activity of the spoT gene product seems to be inhibited by 6-amino-7-chloro-5,8-dioxoquinoline, because the cells treated with the quinone show all characteristics of the spoT$^-$ mutant. The results of this investigation independently support the assumption that ppGpp is metabolized via forming pppGpp. Very similar conclusions have been drawn from experiments with the inhibitor levallorphan (16,17).

The altered metabolism of ppGpp in quinone-treated cells, mimicking the spoT$^-$ phenotype, could be independent from the primary effect of the quinone on leucyl-tRNA synthetase, but it seems more likely that both phenomena are tightly connected. The only cell-free system, so far described, which is able to metabolize ppGpp effectively has been reported very recent-

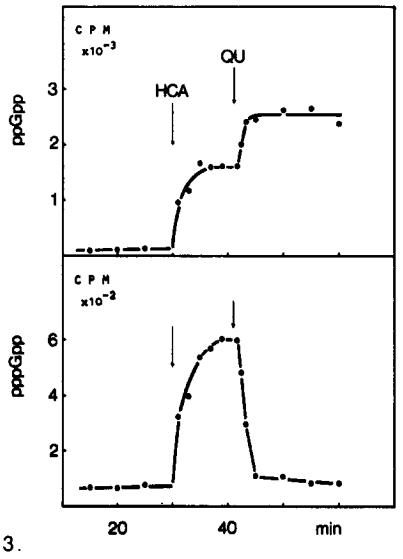

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Figure 3
Effect of 6-amino-7-chloro-5,8-dioxoquinoline on the metabolism of ppGpp and pppGpp in amino acid starved *E. coli* MRE 600.
An exponentially growing culture was labeled with [^{32}P] orthophosphate (80 µCi/ml) before amino acid starvation was imposed by α-hydrazino caproic acid (HCA; 4×10^{-4} M). When ppGpp and pppGpp had reached their steady state level, quinone (QU; 2 µg/ml) was added at the time indicated by the second arrow. Aliquots were withdrawn during all phases of the experiment to determine the relative amounts of ppGpp and pppGpp.

ly by Rapaport and coworkers (7). Lysyl-tRNA synthetase from *E. coli* has been shown to form a mixed nucleotide (ApppGpp) from ppGpp and lysyl-AMP in a reaction analogous to the ATP:pyrophosphate exchange reaction. This activity of aminoacyl-tRNA synthetases has been suggested to constitute a step in the catabolism of ppGpp in *E. coli*. Any inhibition of this activity then should cause an overproduction of ppGpp and a prolongation of its half-life. The tested quinone inhibits the pyrophosphate exchange reaction of leucyl-tRNA synthetase from *E. coli* (Fig. 4). Two characteristics of the altered metabolism of ppGpp in quinone-treated cells could thus be explained by this interaction. The third characteristic of quinone-treated or spoT$^-$ cells, i. e. the lack of pppGpp, has led to the assumption that ppGpp must be converted to the pentaphosphate (5,6). To reconcile this postulation

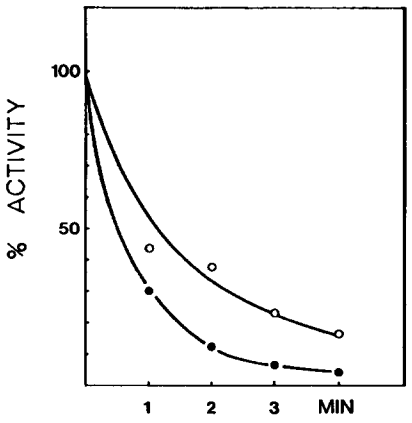

Figure 4
Inactivation of leucyl-tRNA synthetase from *E. coli* MRE 600 by 6-amino-7-chloro-5,8-dioxoquinoline.
10 µg of enzyme were incubated with 2 µg of quinone at 37° C in 200 µl of buffer (HEPES 100 mM, pH 8.15; Mg-acetate 20 mM; NH_4Cl 5 mM). After 1,2,3 and 4 minutes the substrates were added in a volume of 50 µl, containing 2-mercaptoethanol (5 mM).
Substrates for the ATP-pyrophosphate exchange reaction were ATP (final concentr. 4 mM), leucine (2 mM) and [^{32}P] pyrophosphate (2 mM; 4 mCi/mmole). To determine enzyme activity aliquots of 50 µl were withdrawn at 20-seconds-intervals, and the amount of [^{32}P]ATP formed was determined by the charcoal-filter method (18).
Substrates for the leucyl-tRNA forming reaction were ATP (4 mM), [^{14}C] leucine (6 µM; 348 mCi/mmole) and crude tRNA (200 µg). The activity was determined analogously as for the ATP:PPi exchange reaction; filter paper disks were used instead of charcoal filters.
The residual activity of the ATP:PPi exchange (o —— o) and of leucyl-tRNA forming reaction (● —— ●) were plotted versus time of incubation with the quinone. The activities of control assays without quinone were set as 100 %.

with the proposed reaction of aminoacyl-tRNA synthetase, one must assume a mixed nucleotide to be an intermediate in the metabolic conversion of ppGpp to pppGpp.

The small increase of guanosine pentaphosphate during the first minutes after the treatment with quinone could be explained by the still incomplete inhibition of leucyl-tRNA synthetase during this period.

ACKNOWLEDGEMENT

I am indebted to Prof. W. Kersten and to Miss K. Wiebauer for helpful discussions and to Miss M. Kraus for technical assistance. The studies were supported by the Bundesministerium für Forschung und Technologie, Grant SN 6052.

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