DOES FORMYLATION OF INITIATOR tRNA ACT AS A REGULATORY SIGNAL IN *E. COLI*?

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1. Introduction

Since the pioneering studies of Clark and Marcker [1], Capecchi and Eisenstadt [2] and Lengyel [3], it has been generally accepted that initiation of protein synthesis in prokaryotic cells would always involve formylated Met-$tRNA_F^{Met}$ as an initiator. It has, however, been shown [1, 10] that $tRNA_F^{Met}$ differs in its structure from $tRNA_M^{Met}$ (which does not initiate but transfer methionine inside a polypeptidic chain) and this raises the puzzling question: why should there be two different signals (NH_2 blocking and initiator tRNA structure) at the very beginning of protein synthesis in prokaryotic cells? A tentative answer is that a highly evolved regulatory mechanism may be working at the initiation step of protein synthesis to couple it to DNA and RNA syntheses in some crucial events, such as mitosis for instance. The universality of NH_2 blocked methionine at the initiation of prokaryotic protein synthesis has already been questioned by Samuel et al. [4] with *Streptococcus faecalis* and recently by White and Bayley [5] with *Halobacterium cutirubrum*; these authors have found conditions in which cells may grow without formylation of initiator methionine-tRNA.

The formyl residue of formyl methionine derives from the one-carbon pool metabolism; and since the antimitotic trimethoprim [4] is a well known inhibitor of this metabolic pathway it has first been used to investigate the coupling between mRNA synthesis and initiation of protein synthesis on wild type *E. coli*. It is suggested that mRNA synthesis, at least for the lac operon, is probably blocked by trimethoprim before (or in parallel with) protein synthesis, with a correlative accumulation of F-Met $tRNA_F^{Met}$. In addition it was attempted to isolate mutants which would be able to grow without formylation of initiator methionine tRNA. Therefore selection was performed on a medium supplemented with the one-carbon pool metabolites, in the presence of trimethoprim and sulfanilamide (which inhibits folate synthesis). One observes that mutants which grow in this medium fall in several classes involving DNA synthesis (thymine requirement), RNA synthesis (resistance to rifampicin) and protein synthesis (resistance to streptomycin or spectinomycin). Under these conditions it is shown that formylation of Met $tRNA_F^{Met}$ occurs at a low level; this suggests that formylation may act as a coupling signal between replication, transcription and translation.

2. Materials and methods

E. coli strains are derived from K12 (thiamine auxotrophs). They are:

Hfr: Kl 16 K 12 3000
Kl 161 (thy^-) K 12 3300 ($lac\ i^-$)
C 234 (pab^-)

F^-:
600 C (thr^- leu^-)
2001 C (thr^- leu^- str^r)
PA_1 (Arg^- rel^- str^r)
PA_2 (Arg^- rel^+ str^r) [6]

Trimethoprim was chosen as a dihydrofolate reductase inhibitor because it has been shown [7] that it is a most potent inhibitor in K12 strains: it penetrates the cells much more easily than other folate antagonists.

Table 1
Met tRNA and FMet tRNA content of trimethoprim treated cells.

	Kl 16						Kl 161 (thy⁻)	
Trimethoprim (μg/ml)	0	0.15	0.30	0.75	1.50	3.75	0	7.5
Met tRNA pM/mg wet cells	1.80	2.15	2.25	2.75	3.40	3.35	1.75	1.85
FMet tRNA pM/mg wet cells	3.20	2.90	3.65	4.55	5.05	5.05	3.70	3.90
FMet/Met + FMet %	64	57	62	62	60	60	68	68
Generation time minutes	75	86	120	175	240	310	70	73

Cells are grown at 35°C in MFS and aerated by shaking 60 min after trimethoprim addition. tRNA is extracted as described by Marcker [8] (early logphase). In this experiment Met is separated from FMet by enzymatic discharge using crude *E. coli* tRNA ligases and excess AMP and pyrophosphate [9]. Met is counted as [^{3}H]Met (10 Ci/mM).

Culture media were: M 63 [8] supplemented with thiamine (M 63 B_1) and glucose (4 g/ℓ) or glycerol (6 g/ℓ) and the required metabolites for auxotrophic strains; MLFR which is M 63 B_1 supplemented with 12 g/ℓ Bacto Folic Assay medium (Difco); 200 mg/ℓ serine, glycine, methionine; 80 mg/ℓ thymine; 10 mg/ℓ adenine, guanine and 4 g/ℓ glycerol; MFS which is M 63 B_1 supplemented with 200 mg/ℓ serine, glycine, methionine 80 mg/ℓ thymine; 10 mg/ℓ uridine, 10 mg/ℓ adenine, guanine; 1 mg/ℓ pantothenic acid (calcium salt).

Mutagenesis was induced on exponentially growing cultures (5×10^7 cells/ml) with ethyl methane sulfonate (EMS) [8] and selection for resistant mutants was obtained on 15% agar plates containing MLFR supplemented with 50 mg/ℓ trimethoprim and 1 g/ℓ sulfanilamide.

In some cases rifampicin (50 μg/ml), streptomycin (100 μg/ml) or spectinomycin (Upjohn) (100 μg/ml) were added to test for resistance. Sugar utilization was assayed on EMB plates.

In vivo incorporation of [^{3}H]methionine (10 Ci/mM) was obtained on MFS devoid of methionine and tRNA was phenol extracted after extensive washing of cells in glycerophosphate buffer pH 6.0 (10 mM) $MgCl_2$ (10 mM). The relative amounts of Met and FMet tRNA were measured either by enzymatic discharge in the presence of excess AMP and pyrophosphate [9] or by electrophoresis at pH 3.5 [10] after discharging tRNA in 0.8 M triethylamine for 15 min at 37°C.

β Galactosidase and transacetylase were assayed after cells had been induced with isopropyl βD-thiogalactoside (IPTG) or repressed with thiophenyl βD-galactoside (TPG) with the standard procedures [8] except that transacetylase was assayed in measuring [^{14}C]acetyl IPTG radioactivity from [^{14}C]acetyl CoA instead of using dithionitrobenzoic acid.

3. Results and discussion

3.1. *Trimethoprim effect on wild type E. coli (K 16, K 12 3000, K 12 3300)*

As already described by several investigators [11–13] one observes that DNA synthesis (incorporation of [^{3}H]thymidine), RNA synthesis (incorporation of [^{3}H]uridine) and protein synthesis (incorporation of [^{14}C]leucine or [^{14}C]proline) are reduced to less than 10% of their initial value 3 min after addition of 5 μg/ml trimethoprim (at 37°C, in MFS – devoid of thymine when radioactive thymidine is added – for a generation time of 55 min). In the case of Kl 161 which is a thymine auxotroph, this addition of trimethoprim has no effect; furthermore, protein synthesis proceeds at an almost normal rate for 1 hr when

only a small amount of thymine (5 μg/ml instead of 30 μg/ml) is present in the medium.

The effect of trimethoprim on protein synthesis is generally ascribed to inhibition of formylation of charged initiator tRNA. Therefore the amount of Met-tRNA and FMet-tRNA were measured at different trimethoprim concentrations either by selective discharging or by direct electrophoresis. This latter method yielded, in addition to Met and FMet a small amount (≃ 5%) of dipeptides FMet-aa.

Table 1 sums up the results obtained on Kl 16 and Kl 161. Two main features appear: 1) the relative amount of FMet compared to Met is almost unchanged when the generation time increases four times; 2) the total amount of Met tRNA and FMet tRNA per mg of wet cells shows a 80% increase. Thus trimethoprim either yields an accumulation of charged tRNA or induces an increase in the total amount per cell of $tRNA_F^{Met}$. This may be due to a specific induction of $tRNA_F^{Met}$ synthesis (which would accordingly be less formylated but in a higher concentration) or on several tRNA species including $tRNA_F^{Met}$ and $tRNA_M^{Met}$. Therefore the primary target of trimethoprim inhibition is not protein synthesis; this conclusion is in agreement with Dale and Greenberg [13] and casts doubt on experiments where trimethoprim is used as a specific inhibitor of protein synthesis.

3.2. *Trimethoprim effect on the lac operon expression*

Kennel and Simmons have recently shown [14] with direct investigation of the messenger RNA that there is a coupling between translation and transcription for the lac operon. I have investigated this coupling when trimethoprim is added to the culture, with the help of thiophenyl galactoside (TPG) which is a repressor of lac operon transcription. Although this method is not very precise it suggests that trimethoprim may act more or less directly at the RNA level.

The lac operon is induced by addition of IPTG 1 hr before trimethoprim or other inhibitors are added. When 1 μg/ml of trimethoprim is added to an exponentially growing culture induced with 100 μM IPTG the rate of increase of β galactosidase and transacetylase proceeds at a normal rate for at least 1 hr whereas the generation time has nearly tripled (175 min compared to 60 min). Upon the addition of 5 μg/ml of trimethoprim in a culture induced with 35 μM IPTG

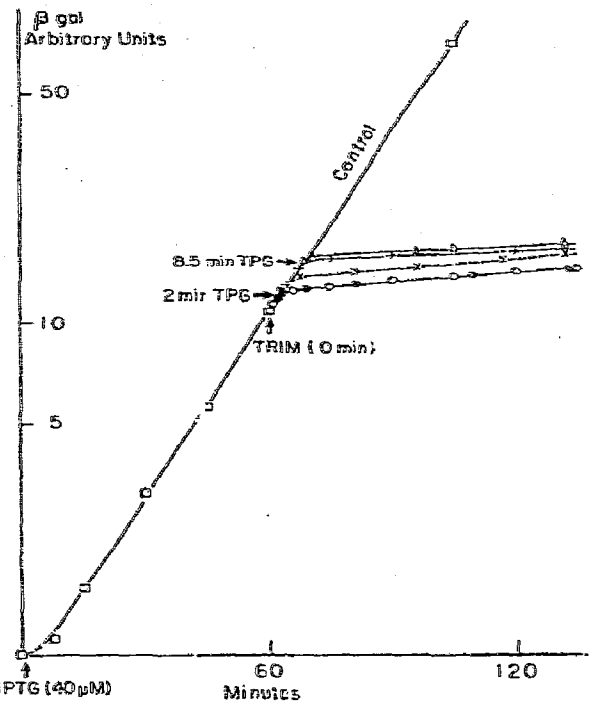

Fig. 1. Effect of trimethoprim on β galactosidase synthesis. *E. coli* Kl 16 was allowed to grow in MFS up to a density of 0.4 (600 nm). IPTG (40 μM) was added and the growth proceeded for 60 min (□—□—□) and control 105 min). At this time (0 min) the culture was separated in 5 samples where trimethoprim (5 μg/ml) and/or TPG (2 mM) were added (+ — + — +) trim (0 min) alone; (● —●—●) trim + TPG (0 min); (X — X — X) trim (0 min) + TPG (2 min); (○—○—○) TPG (0 min) alone (△—△—△) trim (0 min) + TPG (8.5 min). The data for transacetylase are similar but less precise and the slope of the control is slightly higher.

this is no longer the case; after 10 min increase at the normal rate the concentration of these lac operon enzymes levels on. Fig. 1 shows that 2 mM TPG inhibits very efficiently transcription of the lac operon since β galactosidase and transacetylase are no longer formed (or only at a slow rate) after about 4 min which is the likely value for the turnover of the messenger RNA of the lac operon. When the TPG is added 2 min after trimethoprim the rate of increase of the lac operon expression ceases after four more minutes, whereas, after 8.5 min the plateau is attained within less than 2 min. These observations suggest that (at least in the case of the lac operon) trimethoprim has a more or less direct inhibitory effect on RNA synthesis such that one cannot tell that inhibition of protein

synthesis is the cause of inhibition of RNA synthesis in this operon. An alternative explanation is that trimethoprim increases the life time of the messenger RNA by slow inhibition of protein synthesis.

In order to obtain a more precise evidence for this mechanism, strains PA_1 (rel^-) and PA_2 (rel^+) were used, but it was observed that these strains could grow not only when trimethoprim was present but also when sulfanilamide was present (on MLFR)! This observation will be explained hereafter.

3.3. *Properties of trimethoprim + sulfanilamide resistant strains*

The most direct method to obtain information on the function of formylation would be to study a *E. coli* mutant which would grow without formylation of initiator tRNA. It is known [15] that this functional group comes from N10 tetrahydrofolate (THF): a mutant which would grow when THF metabolism is blocked and the necessary (one-carbon)metabolites supplied is hoped to be of the expected kind since FMet $tRNA_F^{Met}$ cannot easily be supplied.

Trimethoprim blocks the dihydrofolate (DHF) reduction to THF whereas sulfanilamide blocks the DHF synthesis; thus a first screening was obtained using Kl 16 and searching for mutants growing on MLFR agar plates supplemented with 50 μg/ml trimethoprim and 1000 μg/ml SA. Since one obtains generally 90% of thy^- mutants the screening used a replica-plating step on M 63 glucose plates where thy^- strains cannot grow. Mutants growing on M 63 glucose supplemented with trimethoprim are considered here since those mutants are either permeability mutants or dihydrofolate reductase mutants and probably not directly involving initiation of protein synthesis. Strains which grew on MLFR + Trim + SA, on M 63 glu and not on M 63 + Trim were kept. Five of them were tested for Met and FMet tRNA content when grown on MLFR + Trim + SA after phenol extraction of tRNA, triethylamine discharge and high voltage electrophoresis. In each case it was found that formylation occurred at a level almost normal (about 45% FMet/Met + FMet) whereas in the control this level was reduced to about 10%. Since this could be explained by enhanced values of formylase activity or increase in some specific rates in the one-carbon pool metabolism, I decided to use as a wild type a *para*-amino benzoic acid auxotroph (C 234) which should be more drastically affected by SA than Kl 16.

Cultures of C 234 were mutagenized with EMS and plated on MLFR + Trim + SA. After 48 hr growth at 37°C, colonies were visible. They were picked up and spotted on the same selective medium for purification and this procedure was repeated twice.

The possible coupling between one-carbon pool metabolism and DNA, RNA and protein syntheses was tested by assaying mutants for thymine requirement, rifampicin resistance and streptomycin or spectinomycin resistance.

The result is as follows: among 1990 (Trim + SA) resistant C 234 mutants *102 rifampicin resistant, 1 spectinomycin resistant* and *1 streptomycin resistant* (these don't cross resist!) were found. Since no Str^r, Spc^r or Rif^r due to permeability have been found yet, those mutants are most probably located at the spc (63 min) str (64 min) and rif (77 min) loci; indeed the converse experiment i.e. the use of Str^r, Spc^r or Rif^r mutants selected in the normal way (i.e. on the corresponding antibiotic) yields cultures which grow on MLFR + Trim + SA: this is the reason why PA_1 and PA_2 grow since on this medium they were found to be Str^r.

The total mutagenesis yield is about 2×10^{-4} which gives a yield of 10^{-5} for rif and 10^{-7} for str and spc. This supports the idea that almost all Rif^r, Spc^r and Str^r have thus been selected since their relative occurrence, when obtained by selection on the cognate antibiotic is similar (spontaneous: Rif^r 5×10^{-7} Spc^r, Str^r 5×10^{-9}). Despite this likely interpretation these resistant strains may be traced as very improbable double mutations; therefore the Met tRNA and FMet tRNA content were not measured on strains selected on trim + SA but on strains obtained from selection with the cognate antibiotic (strain derived from 600C). One finds that the FMet content is reduced to less than 15% of that of the control when 50 μg/ml trimethoprim and 1000 μg/ml are added to the culture (after a 60 min incubation in MFS containing trim + SA, filtration, reincubation for 3 min with [^{3}H]methionine and finally addition of 100 μg/ml chloramphenicol). One should note at this point that the FMet content was not found to be reduced to zero.

Among other mutants many (90%) are thy^- but some grow on glu + Paba and some are mal^-. A more general characterization is in progress. Enrichment in

Str^r and Spc^r mutants has been further tested by incubating about 10^{10} bacteria (Kl 16 or K12 3000) in MLFR + TMP + SA then diluting after 48 hr growth in fresh media and finally repeating this procedure, spreading 10^7 bacteria on streptomycin or spectinomycin plates. From the spontaneous Str^r and Spc^r mutation rates one should not obtain any living colonies; it is, on the contrary, observed that one finds many such colonies!

Thus it seems likely that the changes in the 30 S ribosomal subunits due to Spc^r or Str^r yields bacteria able to grow with Met $tRNA_F^{Met}$. The *in vitro* assay to test this hypothesis is in progress. The rifampicin resistance is more difficult to explain since it involves the β subunit of DNA dependent RNA polymerase. This subunit is probably somehow coupled to initiation of protein synthesis either directly or because the mRNA or rRNA structures are affected (which seems rather unlikely). This phenomenon may be related to the experiments presented above on lac operon and Imamoto and Tani experiments showing diversity of regulation of genetic transcription [16].

4. Conclusion

Some of the gene modifications which more or less reverse growth inhibition due to blocking of the one-carbon pool metabolism (in the presence of the necessary metabolites) are the thy^- mutation, the rifampicin resistance and the streptomycin or spectinomycin resistance. These are related to DNA synthesis, RNA synthesis and protein synthesis. One may therefore think that there is an underlying mechanism which accounts for the corresponding coupling between these syntheses. I wish to add a weak teleonomic argument for situating this coupling in the one-carbon pool metabolism itself, not only because of formylation of initiator tRNA (one may also think of a specific modification of tRNA itself) but because of the very presence of thymine in DNA.

It appears to me that living beings had to loose a rather important genetic stability when thymine was introduced as an informational base: adenine, uracil, cytosine and guanine were most probably selected among structurally similar molecules not only because of their ability to pair by hydrogen bonds but because they are *non fluorescent* (no stable excited state of low energy) at ambient temperature. Accordingly they are almost non reactive with one another, even when stacked; contrarywise it is well known that thymine (which is slightly fluorescent) yields dimers in DNA at a so high frequency (for genetic stability) that an excision—repair mechanism has been selected to reduce the effect of this phenomenon. Thus the introduction of a methyl group in a DNA base must have been of prime importance to overcome its disadvantages. There are two ways for methylation in the cell, either use of S-adenosyl methionine or use of the one-carbon pool. Not only does the CH_3 of thymine come from the latter but it does not use the tetrahydrofolate coenzyme in catalytic but in *stoichiometric* amounts. This is a very stringent coupling to the general cellular metabolism and this gives a central role to the dihydrofolate reductase. It therefore supports the idea that the tetrahydrofolate metabolism is crucial for a coupling between replication, transcription and translation and thus to mitosis.

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