Communication

Formylation Is Not Essential for Initiation of Protein Synthesis in All Eubacteria

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Formylation of the initiator methionyl-tRNA, catalyzed by methionyl-tRNA formyltransferase, has long been regarded as essential for initiation of protein synthesis in eubacteria. Here, we show that this process is, in fact, dispensable in Pseudomonas aeruginosa. Disruption of the chromosomal methionyl-tRNA formyltransferase gene in P. aeruginosa resulted only in a moderate decrease in the rate of cell growth, whereas in Escherichia coli cell growth was severely impaired. The ability of the P. aeruginosa mutant strain to grow was not due to an additional copy of the methionyl-tRNA formyltransferase gene or to N-acetylation of the methionyl moiety by a group other than formyl. These results indicate that P. aeruginosa can carry out formylation-independent initiation of protein synthesis, using the nonformylated methionyl-tRNA. Therefore, the dogma that eubacteria require formylation of the initiator methionyl-tRNA for initiation of protein synthesis may have been an invalid generalization of results obtained with E. coli.

Field synthesis in eubacteria, and in the chloroplasts and mitochondria of eukaryotes, can be initiated using the initiator formyl-methionyl-tRNA (fMet-tRNA(fMet)) (1). Formylation is specific for the initiator methionyl-tRNA (Met-tRNA(fMet)) and is catalyzed by methionyl-tRNA formyltransferase (MTF), which is encoded by the fmt gene (2–5). The features in the Escherichia coli tRNA(fMet) required for formylation are the base-base mismatch between nucleotides 1 and 72 and the second and third base pairs of the acceptor stem (4–6). The MTF moiety allows initiation factor IF-2 to recognize the initiator tRNA and reject other tRNAs. This conclusion is based on in vivo analysis of the effect of overproduction of IF-2 on the activity of E. coli tRNA(fMet) mutants defective in formylation (7, 8) and on in vitro studies of IF-2 interaction with N-blocked aminoacyl-tRNA (9, 10). The formyl group also prevents the tRNA(fMet) from partici-Optation in elongation by blocking binding of elongation factor EF-Tu (5, 7).

Formylation of the Met-tRNA(fMet) is generally accepted as a key checkpoint required for initiation of protein synthesis in eubacteria. This dogma, based primarily on studies conducted in E. coli, was further substantiated by the finding that disruption of the E. coli chromosomal fmt gene severely curtailed cell growth (3). However, some earlier studies have obtained circumstantial evidence that questioned this generalization. A Streptococcus faecalis folate-deficient strain, which is unable to synthesize the formyl donor N10-formyltetrahydrofolate (fTHF), was shown to be unaffected in growth or viability (11). However, it was not clear that S. faecalis can initiate protein synthesis in the absence of formylation, since the strain also incurred additional chromosomal mutations that affect proper tRNA modification (11).

While the studies in S. faecalis were inconclusive, they hinted that formylation may not be a prerequisite for initiation of protein synthesis in all eubacteria. Therefore, we investigated the significance of the formylation step in Pseudomonas aeruginosa, a Gram-negative, opportunistic pathogen that is distantly related to E. coli. The results show that the process of initiation of protein synthesis in P. aeruginosa is not strictly dependent on formylation and can accommodate the use of non-N-blocked Met-tRNA(fMet). The extent to which other eubacteria have this capacity to initiate translation thus becomes an open question.

EXPERIMENTAL PROCEDURES

Materials—The plasmids pEX100T (12), pUCGM (13), and pUCP26 (14) and the P. aeruginosa PA01 strain were provided by Dr. J. S. Lam, Department of Microbiology, University of Guelph. The pACTN vector was described previously (15). The E. coli methionyl-tRNA synthetase (MetRS) and initiator tRNA (15) and P. aeruginosa MTF (15, 16) were overproduced and purified as described. The fmt-deficient strain of E. coli was constructed previously (16).

Construct for Expression of the P. aeruginosa and E. coli MTFs in the E. coli fmt Mutant Strain—The open reading frame (ORF) of the fmt genes was cloned into the Ncol and BamHI sites in pACTN (15). In this construct, the fmt genes are under the control of the Lac promoter and the Shine-Dalgarno and transcription termination sequences of gene 10 of bacteriophage T7.

Construct for Expression of the P. aeruginosa MTF in the P. aeruginosa fmt Mutant Strain—A 1.4-kilobase pair BamHI fragment containing the wild type P. aeruginosa fmt gene was inserted into the same site in the P. aeruginosa shuttle vector pUCP26. The cloned fragment is under the control of the Lac promoter.

Disruption of the Chromosomal P. aeruginosa fmt Gene—The P. aeruginosa fmt gene on a 6-kilobase pair fragment was isolated previously from a genomic library (GenBank accession number AF073952) (16). A 4.5-kilobase pair SmalI segment containing the fmt gene and flanking sequences was obtained from the 6-kilobase pair fragment and inserted into the same site of pEX100T. An 840-base pair fragment containing the gentamycin resistance gene (GmR) was obtained by PCR amplification using pUCGM as the template. The fragment was digested with MfeI and inserted into the same site in the fmt gene on pEX100T. The pEX100T/fm:GmR construct was used to replace the chromosomal MTF gene of the P. aeruginosa PA01 strain (12). Gene replacement was verified by PCR analysis using primers complementary to the 5′ and 3′ ends of the fmt ORF. The sequence of the primers is 5′-TGTTCGATGCGGATGACGC-GCGACGCGC-3′ and 5′-TTTGC-GATCCATTGACCACGAGCTG-3′.

Preparation of Cell Extracts—The wild type and mutant fmt P. aeruginosa strains harboring the pUCP26 vector without the fmt gene were grown overnight at 37 °C in Luria-Bertani medium supplemented with 60 μg/ml tetracycline. An aliquot of the cultures were diluted in...
50-fold into 3 ml of medium containing tetracycline and grown for 3 h at 37 °C. Cells from 1.2 ml of culture was pelleted by centrifugation and lysed (8). The cell lysate was centrifuged and an aliquot of the supernatant was mixed with 3 volumes of 25.6 mM Tris-HCl, pH 8.0, 13.5 mM β-mercaptoethanol, 200 mM KCl, and 68% glycerol (w/v) and stored at −20°C (8).

Measurement of MTF Activity—The initiator tRNA substrate was acetylated with methionine at 37 °C for 30 min. The incubation mixture (20 μl) consisted of 20 mM imidazole HCl, pH 7.5, 150 mM NH₄Cl, 10 mM MgCl₂, 0.1 mM EDTA, 10 μg/ml bovine serum albumin, 100 μM methionine (specific activity: 13,000–15,000 cpm/pmol), 2 mM ATP, 5 μM tRNAfMet, and an excess of MetRS (1 μg). To measure the fo- 

In vitro studies of protein synthesis using extracts from E. coli showed that translation was stimulated by fMet-tRNAfMet by using trimethoprim to impair folate metabolism caused the cells to grow very slowly (18–20). These early studies led to the belief that formylation is an important prerequisite for initiation of protein synthesis in eubacteria. Recently, this view was supported by the finding that inactivation of the E. coli chromosomal fmt gene, which codes for the formylating enzyme, severely curtailed cell growth (3). Both in vitro and in vivo studies showed that the formyl group is important for selection of the initiator tRNA by IF-2 at the initiation site of the ribosome and for preventing misappropriation of the tRNA in elongation (5, 7–10).

Some circumstantial evidence obtained from studies conducted in S. faecalis and Bacillus subtilis questioned the importance of formylation in initiation of protein synthesis in eubacteria. In contrast to E. coli, B. subtilis was capable of growth when formylation was blocked by trimethoprim inhibition of folate metabolism (21). Similarly, a S. faecalis mutant deficient in folate synthesis was not affected in growth (11). In the case of S. faecalis, the selection conditions used to isolate the mutant strain resulted in additional chromosomal mutations, which preceded partial tRNA modification. This observation suggests that growth of B. subtilis could be due to compensatory chromosomal mutations. Therefore, it is not clear whether formylation is required for initiation of protein synthesis in these organisms.

Disruption of the P. aeruginosa Chromosomal MTF Gene—To investigate the importance of MTF-tRNAfMet formylation in P. aeruginosa, the chromosomal fmt gene was replaced with a mutant copy by homologous recombination (12). The fmt gene used in this study was isolated previously from a genomic library by functional complementation of a MTF-deficient E. coli strain (16). A 4.5-kilobase pair Smal segment containing the fmt gene as well as flanking sequences was obtained from the genomic clone and inserted into the same site in the suicide vector pEX100T. The fmt gene was inactivated by insertion of the Gm³ cassette into a MfeI site. P. aeruginosa transformants containing the pEX100T::fmt:Gm³ vector in the chromosome were identified by selecting for carbenicillin and gentamycin resistance. Resolution of the plasmid was achieved by subjecting the merodiploid strain to sucrose counter-selection in the presence of gentamycin.

Several sucrose-intensive and gentamycin-resistant colonies were observed within 24 h of incubation at 37 °C. Replacement of the wild type fmt gene with the mutant copy was verified by PCR analysis using primers complementary to the 5' and 3' ends of the fmt ORF and chromosomal DNA from the merodiploid (lane 1), MTF mutant (lane 2), and wild type (lane 3) strains. The pEX100T::fmt:Gm³ DNA was used as a control (lane 4).
of formylation activity in cell extracts prepared from the wild type and mutant strains (Fig. 3). The total activity in cell extracts prepared from the wild type parental strain increased with the amount of protein assayed. In contrast, no formylation activity was detected in cell extracts prepared from the mutant strain even at high protein levels. These results show that the

P. aeruginosa Has the Capacity to Initiate Protein Synthesis with Nonformylated Met-tRNA$^{fMet}$

We investigated whether N-acylation of the Met-tRNA$^{fMet}$ by a functional group other than the formyl group could account for the growth of the P. aeruginosa fmt mutant strain. Total tRNAs were isolated from the wild type and mutant strains, under conditions that stabilize the aminoacyl ester linkage (3). One portion of the sample was completely deacylated, by incubating the tRNA under alkaline conditions. A second portion was treated with CuSO$_4$, which cleaves the ester bond of aminoacyl-tRNA but not that of N-acyl-Met-tRNA$^{fMet}$. Both tRNA samples were reacylated with methionine, and formyl acceptance was measured using P. aeruginosa MTF overproduced in E. coli and purified by affinity chromatography (15). The amount of N-blocked Met-tRNA$^{fMet}$ in the wild type and mutant MTF strains was determined by the difference between formyl acceptance of the completely deacylated and CuSO$_4$-treated tRNA samples.

P. aeruginosa fmt mutant strain was devoid of formylating activity.

P. aeruginosa Has the Capacity to Initiate Protein Synthesis with Non-N-blocked Met-tRNA$^{fMet}$—We investigated whether N-acylation of the Met-tRNA$^{fMet}$ by a functional group other than the formyl group could account for the growth of the P. aeruginosa fmt mutant strain. Total tRNAs were isolated from the wild type and mutant strains, under conditions that stabilize the aminoacyl ester linkage (3). One portion of the sample was completely deacylated, by incubating the tRNA under alkaline conditions. A second portion was treated with CuSO$_4$, which cleaves the ester bond of aminoacyl-tRNA but not that of N-acyl-Met-tRNA$^{fMet}$. Both tRNA samples were reacylated with methionine, and formyl acceptance was measured using P. aeruginosa MTF overproduced in E. coli and purified by affinity chromatography (15). The amount of N-blocked Met-tRNA$^{fMet}$ in the wild type and mutant MTF strains was determined by the difference between formyl acceptance of the completely deacylated and CuSO$_4$-treated tRNA samples. Based on
this analysis, 80% of the Met-tRNA\textsuperscript{Met} in the wild type strain was N-blocked (Fig. 4). However, only the unblocked Met-tRNA\textsuperscript{Met} species was found in the mutant strain. This result showed that the Met-tRNA\textsuperscript{Met} in the MTF-deficient strain was not N-acetylated by another group. Taken altogether, the results indicate that protein synthesis in the MTF-deficient \textit{P. aeruginosa} strain is initiated with the initiator methionyl-tRNA and not with N-blocked Met-tRNA\textsuperscript{Met}.

In archaebacteria and the cytoplasm of eukaryotes, protein synthesis is initiated with the Met-tRNA (23). These initiator tRNAs contain an A:U base pair which is, in part, required for initiation factor (eIF) recognition of the tRNA (24). In contrast, eubacterial initiator tRNAs contain a C \times A mismatch between nucleotides 1 and 72. The mismatch in the \textit{E. coli} initiator tRNA is a key formylation determinant (4–6). Therefore, for initiation in the absence of formylation, it is possible that \textit{P. aeruginosa} is using an initiator tRNA with functional properties comparable with those of eukaryotes. A search of the \textit{P. aeruginosa} genome sequence data base identified two initiator tRNA genes encoding tRNAs that are almost identical. The sequences of the initiator tRNAs are homologous to the \textit{E. coli} tRNA\textsuperscript{Met} (Fig. 5). Moreover, the \textit{P. aeruginosa} tRNAs contain a C \times A mismatch between nucleotides 1 and 72. This feature was used by the \textit{P. aeruginosa} MTF to recognize the initiator tRNA, since overproduction of the enzyme increased the initiator activity of a formylation-defective tRNA\textsuperscript{Met} mutant with a base pair between nucleotides 1 and 72 (data not shown). These results suggest that \textit{P. aeruginosa} is using the tRNA\textsuperscript{Met} and not a unique initiator tRNA to initiate protein synthesis in the absence of formylation.

IF-2 is essential for initiation of protein synthesis in \textit{E. coli} (25). The protein has a higher affinity for the fMet-tRNA\textsuperscript{Met} than for the Met-tRNA\textsuperscript{Met}, indicating that the formyl group is an IF-2 recognition element (9, 10). \textit{In vitro} studies showed that IF-2 selects the fMet-tRNA\textsuperscript{Met} at the initiation site of the preinitiation complex (9). Therefore, in \textit{E. coli}, IF-2 is partly responsible for excluding the participation of the nonformylated Met-tRNA\textsuperscript{Met} in initiation. This is consistent with the observation that in the MTF-deficient \textit{E. coli} strain, initiation of protein synthesis is severely impaired. The eIF-2s of archaeobacteria and eukaryotic cytoplasm are involved in selecting the initiator Met-tRNA for utilization in initiation of translation. A BLAST search of the \textit{P. aeruginosa} genome data base identified sequences that were homologous to the \textit{E. coli} IF-2 gene but none that were homologous for the eIF-2 gene. Therefore, it is conceivable that the \textit{P. aeruginosa} IF-2 has dual substrate specificity and facilitates utilization of both Met-tRNA\textsuperscript{Met} and fMet-tRNA\textsuperscript{Met} in initiation or that an unidentified initiation factor, which only recognizes the Met-tRNA\textsuperscript{Met} species, is involved.

We have shown that \textit{P. aeruginosa} can carry out formylation-independent initiation of protein synthesis, using the Met-tRNA\textsuperscript{Met}. This finding represents the first direct evidence that eubacteria have the ability to initiate translation with fMet-tRNA\textsuperscript{Met} and Met-tRNA\textsuperscript{Met}. While it is not known whether this phenomenon is unique to \textit{P. aeruginosa}, it opens the possibility that other eubacteria may have the capacity to use both forms of the tRNA\textsuperscript{Met} for initiation. The availability of various \textit{fmt} sequences will facilitate investigation of this question in both Gram-positive and Gram-negative bacteria.

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