The *Pseudomonas aeruginosa* Initiation Factor IF-2 Is Responsible for Formylation-independent Protein Initiation in *P. aeruginosa*

Received for publication, July 16, 2004, and in revised form, September 2, 2004 Published, JBC Papers in Press, September 22, 2004, DOI 10.1074/jbc.M408086200

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Formylation of the initiator methionyl-tRNA (Met-tRNAfMet) was generally thought to be essential for initiation of protein synthesis in all eubacteria based on studies conducted primarily in *Escherichia coli*. However, this view of eubacterial protein initiation has changed because some bacteria have been demonstrated to have the capacity to initiate protein synthesis with the unformylated Met-tRNAfMet. Here we show that the *Pseudomonas aeruginosa* initiation factor IF-2 is required for formylation-independent protein initiation in *P. aeruginosa*, the first bacterium shown to have the ability to initiate protein synthesis with both the initiator formyl-methionyl-tRNA (fMet-tRNAfMet) and Met-tRNAfMet. The *E. coli* IF-2, which participates exclusively in formylation-dependent protein initiation in *E. coli*, was unable to facilitate utilization of Met-tRNAfMet in initiation in *P. aeruginosa*. However, the *E. coli* IF-2 was made to function in formylation-independent protein initiation in *P. aeruginosa* by decreasing the positive charge potential of the cleft that binds the amino end of the amino acid attached to the tRNA. Furthermore increasing the positive charge potential of this cleft in the *P. aeruginosa* IF-2 prevented the protein from participating in formylation-independent protein initiation. Thus, this is the first demonstration of a eubacterial IF-2 with an inherent capacity to facilitate utilization of Met-tRNAfMet in protein initiation, discounting the dogma that eubacterial IF-2 can only allow the use of fMet-tRNAfMet in protein initiation. Furthermore these findings give important clues to the basis for discriminating the initiator Met-tRNA by IF-2 and for the evolution of alternative mechanisms for discrimination.

The initiator methionyl-tRNA is used to initiate protein synthesis in Archaeabacteria and the cytoplasm of eukaryotes. In contrast, protein synthesis in eubacteria and in certain eukaryotic organelles, such as mitochondria and chloroplasts, can be initiated using the initiator formyl-methionyl-tRNA. Formylation is specific for the initiator methionyl-tRNA and is catalyzed by methionyl-tRNA formyltransferase (MTF)¹ (1–6). *In vitro* studies of protein synthesis using extracts from *Escherichia coli* showed that translation was stimulated by fMet-tRNAfMet (7). Furthermore inhibition of formylation in *E. coli* by using trimethoprim to impair folate metabolism caused the cells to grow very slowly. These early studies led to the general belief that formylation of the Met-tRNAfMet is a key step in initiation of protein synthesis in all eubacteria. This view was reinforced by the findings that disruption of the MTF gene in *E. coli* (8), *Streptococcus pneumoniae* (9), and *Bacillus subtilis* (10) severely curtailed cell growth, and the MTF gene is present in all eubacterial genomes sequenced to date. However, this dogma of eubacterial protein initiation is no longer valid because some bacteria have been shown to initiate protein synthesis independently of formylation (11–14).

*Pseudomonas aeruginosa* was the first bacterium shown to have the capacity to use Met-tRNAfMet to initiate protein synthesis (12). Subsequently it was demonstrated that formylation of Met-tRNAfMet was not necessary either for protein initiation in *Staphylococcus aureus* (11), *Haemophilus influenzae* (15), and *Saccharomyces cerevisiae* mitochondria under certain growth conditions (16–18). *Streptococcus faecalis* is another bacterium that may also be capable of formylation-independent protein initiation based on evidence obtained from early studies of a *S. faecalis* mutant strain unable to synthesize formyltetrahydrofolate, the formyl donor in the Met-tRNAfMet formylation reaction (13, 19). Thus, unlike *E. coli*, *S. pneumoniae* and *B. subtilis*, which are dependent on fMet-tRNAfMet for protein initiation, *P. aeruginosa*, *S. aureus*, *H. influenzae*, and possibly *S. faecalis* can perform initiation with either fMet-tRNAfMet or Met-tRNAfMet. The mechanism, however, that allows utilization of Met-tRNAfMet in protein initiation in these bacteria is not understood. Furthermore why some bacteria have the ability to initiate protein synthesis with both fMet-tRNAfMet and Met-tRNAfMet and others with only Met-tRNAfMet is not understood.

The mechanism that facilitates the use of fMet-tRNAfMet in bacteria that require formylation for protein initiation is better understood and is absolutely dependent on the initiation factor IF-2 (20, 21). IF-2 selects the fMet-tRNAfMet over Met-tRNAfMet in protein initiation, reinforcing the view of eubacterial protein initiation has changed because some bacteria have the ability to initiate protein synthesis independently of formylation (11–14).

The abbreviations used are: MTF, methionyl-tRNA formyltransferase; Met-tRNAfMet, initiator methionyl-tRNA; fMet-tRNAfMet, initiator formyl-methionyl-tRNA; fMet, formyl-methionyl; IF, initiation factor; EF, elongation factor.

* This work was supported by a discovery grant from the Natural Sciences and Engineering Research Council of Canada (NSERC) (to D. M.) and an operating grant from the Canadian Institutes of Health Research (to C. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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lation-deficient \textit{P. aeruginosa} strain. In contrast, the \textit{E. coli} IF-2 was unable to facilitate the use of Met-tRNA\textsuperscript{Met} in protein initiation in \textit{P. aeruginosa}. It is, therefore, likely that IF-2 allows formylation-independent protein initiation in other eubacteria that have the capacity to initiate protein synthesis with Met-tRNA\textsuperscript{Met}. A structural model of the tRNA-binding domains of the \textit{P. aeruginosa} and \textit{E. coli} IF-2s was established using the solution structure of the \textit{Bacillus steatorrhophilus} IF-2 tRNA-binding domain (27). This structure is similar to the crystal structure of the \textit{Thermus aquaticus} elongation factor EF-Tu. The co-crystal structure of the \textit{T. aquaticus} EF-Tu complexed with the \textit{E. coli} cysteinyl-tRNA\textsuperscript{Cy3} has also been determined (28), allowing superimposition of the tRNA onto the tRNA-binding sites of the \textit{P. aeruginosa} and \textit{E. coli} IF-2s. Analysis of the IF-2 structures indicated that the cleft containing the amino end of the amino acid attached to the tRNA is positively charged. However, this charge is larger for the amino acid attached to the tRNA is positively charged. How-

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Disruption of the Chromosomal \textit{P. aeruginosa} inB\textsuperscript{Gm} Gene—An EcoRI-B\textsuperscript{Xba}I fragment containing the coding sequence of the \textit{P. aeruginosa} inB\textsuperscript{Gm} gene was obtained from pUCP261PIF2 and ligated into the same sites in the plasmid. The inB\textsuperscript{Gm} gene is under the control of the lacZ promoter in the plasmid. An 840-base pair fragment containing the gentamycin resistance gene (\textit{Gm}\textsuperscript{Gm}) was cloned into the KpnI site of the inB\textsuperscript{Gm} gene in pEX18AP\textsuperscript{Akat}, generating pEX18AP\textsuperscript{Kat}/B\textsuperscript{Gm}. The chromosomal inB\textsuperscript{Gm} gene in the \textit{P. aeruginosa} PA01 strain harboring pUCP1PIF2 was replaced using the pEX18AP\textsuperscript{Kat}/B\textsuperscript{Gm} construct. Gene replacement was verified by PCR analysis using the following primers: P1, 5’-GGG AGC GAG AGA CTC CAG CTC CTC-3’, P2, 5’-AGG AAC CAG TGC TGC CCA CGA-3’, P3, 5’-GTT ACG CAG CAG GGC AGT CGC-3’, P4, 5’-GGC GGT AGC CGA GAT CG-3’. Preparation of Cell Extracts for Western Blot Analyses—The wild type and mutant \textit{P. aeruginosa} strains harboring pUC26 without or with the \textit{P. aeruginosa} fmt gene were grown overnight at 37 °C in Luria-Bertani medium supplemented with 60 \mu M tetracycline (12). An aliquot of the culture was diluted into 3 ml of fresh medium containing tetracycline and grown for 3–5 h at 37 °C. Cells from 1.2 ml of culture were pelleted by centrifugation and lysed (12). The cell lysate was centrifuged, and an aliquot of the supernatant (6 \mu g of total protein) was subjected to SDS-PAGE on a 10% polyacrylamide gel. The separated proteins were transferred electrophoretically onto polyvinylidene difluoride membrane. Western blot analysis was performed using the enhanced chemiluminescence protocol as described in the supplier.

\textbf{Materials and Methods.} The plasmids pEX18AP, pUCGM, pUCP26, pACTN, and pUCP26-PAMTF, the \textit{P. aeruginosa} fmt mutant strain; and \textit{P. aeruginosa} PA01 were kindly provided (12). Rabbit anti-IF-2 was provided by Dr. U. L. Rajbhandari, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA. \textbf{Constructions for the Expression of the \textit{P. aeruginosa} and \textit{E. coli} IF-2s in \textit{P. aeruginosa} fmt Mutant Strain—A 3.5-kilobase pair fragment containing the \textit{P. aeruginosa} inB\textsuperscript{Gm} gene encoding IF-2 was prepared by PCR using \textit{P. aeruginosa} chromosomal DNA as the template and the oligonucleotides 5’-GCT GGT GGA TCC GCC ACG ACG ACG CTC CAG CCG TCT-3’ and 5’-ACC ACC GAC TCC GCC AGT TGA CCG GAT GGA CCC-3’; the fragment was ligated with BamHI and cloned into the same site in pUCP26 to produce pUCP1PIF2. A 2.5-kilobase pair fragment of the coding sequence of the \textit{P. aeruginosa} inB\textsuperscript{Gm} gene was isolated by PCR using the pUCP1PIF2 plasmid as a template and 5’-TTG TGA ATT CTC ATG AGC CAA GTA ATC GAA GAT GAA ACC CCG GGT ACC TCT ATG ATT CTC ATG AGC CAA GTA at the KpnI site in the multiple cloning site. The fragment was ligated with NcoI and BamHI and ligated into the same sites in pACTN. The pACTN-ECIF2 construct was used as a template to prepare an EcoRI-BamHI fragment of the \textit{E. coli} inB\textsuperscript{Gm} gene by PCR. The primers used were 5’-ACC ACC GAA TTC ATG ACA GAT GTA ACG ATT AA-3’ and 5’-GCT GGT GGA TCC TTA AGC AAT GGT ACG TTG GAT-3’, the fragment was cloned into the same sites in pUCP26.

\textbf{RESULTS AND DISCUSSION.} Overexpression of the \textit{P. aeruginosa} IF-2Compensates for the Loss of Formylation in \textit{P. aeruginosa}—Bacteria such as \textit{E. coli}, which are primarily dependent on formylation-dependent protein initiation, are unable to initiate protein synthesis without the activity of IF-2. This has been exemplified by the finding that overexpression of the \textit{E. coli} IF-2 did not alleviate the severe growth defect of an \textit{E. coli} mutant strain lacking a functional MTF (23). However, overexpression of IF-2 has been shown to increase the initiator activity of a formylation-defective \textit{E. coli} initiator tRNA mutant in vivo, suggesting that IF-2 may be able to facilitate utilization of Met-tRNA\textsuperscript{Met} in protein initiation in \textit{E. coli} (23). Therefore, it is possible that protein initiation using the unformylated Met-tRNA\textsuperscript{Met} in the \textit{P. aeruginosa} MTF mutant strain is due to increased expres-
sion of the infB gene encoding IF-2. To investigate this possibility, Western blot analysis was used to assess the IF-2 level in total extract from the wild type and mutant P. aeruginosa strains (Fig. 1). The amount of IF-2 is about the same in the MTF mutant strain harboring the pUCP26 vector without (lane 3) or with the P. aeruginosa fmt gene encoding MTF (lane 4). This is comparable to the IF-2 level in the wild type strain carrying pUCP26 without (lane 1) or with the fmt gene (lane 2). Thus, increased expression of the IF-2 gene is not the reason why the P. aeruginosa MTF mutant strain can initiate protein synthesis without formylation. However, findings presented below suggest that the P. aeruginosa IF-2 is responsible for formylation-independent protein initiation in the MTF mutant strain.

We have established previously that growth of the P. aeruginosa MTF mutant strain on rich medium at 37 °C is only slightly slower compared with the wild type strain (12). However, on minimal medium, the mutant strain (Fig. 2, left panel, sector 2) was found to grow significantly slower than the wild type strain within 15 h of incubation (Fig. 2, left panel, sector 3). Growth of the MTF mutant strain on minimal medium was observed when the incubation was continued for another 33 h (Fig. 2, right panel, sector 2), indicating that the P. aeruginosa MTF mutant strain is viable. We have proposed previously that the P. aeruginosa IF-2 has dual substrate specificity and is responsible for formylation-independent protein initiation in P. aeruginosa (12). To test this notion, growth of the P. aeruginosa MTF mutant strain on minimal medium was used to assess whether overproduction of the P. aeruginosa IF-2 facilitates the use of unformylated Met-tRNA^{Met} in protein initiation (Fig. 2). The wild type P. aeruginosa strains carrying the pUCP26 plasmid without (sector 3) and with the P. aeruginosa fmt (left panel, sector 1) or infB (left panel, sector 4) gene grows on minimal medium within 15 h of incubation. However, no significant growth of the MTF mutant strain harboring pUCP26 alone (left panel, sector 2) was observed within 15 h. In contrast, the mutant strain grows when the P. aeruginosa IF-2 (left panel, sector 5) or MTF (left panel, sector 6) was overproduced. These data suggest that IF-2 is allowing utilization of the initiator Met-tRNA in protein initiation in the P. aeruginosa MTF mutant strain.

To exclude the possibility that overexpression of the P. aeruginosa IF-2 is causing N-acylation of the methionyl moiety of Met-tRNA^{Met} by another group, the N-acylation status of Met-tRNA^{Met} in the P. aeruginosa MTF mutant strain was assessed (Fig. 3). Total RNA, isolated under acidic conditions to prevent hydrolysis of the ester bond between the tRNA and the amino acid, was separated by polyacrylamide gel electrophoresis, and Northern blot analysis was used to detect the tRNA^{Met} (lane 1), fMet-tRNA^{Met}, and Met-tRNA^{Met} species. In the wild type P. aeruginosa strain tRNA^{Met} was entirely present in the fMet form (lanes 2 and 5), whereas only the Met-tRNA^{Met} species was detected in the P. aeruginosa MTF mutant strain (lanes 3 and 8). The fMet form of the RNA was found in the MTF mutant strain overproducing MTF (lanes 4 and 6), but only the Met-tRNA^{Met} species was observed when IF-2 was overproduced (lane 7). These results verify that the P. aeruginosa MTF mutant strain is devoid of MTF and are consistent with IF-2 facilitating formylation-independent protein initiation.

Disruption of the infB Gene in P. aeruginosa Is Lethal—The P. aeruginosa IF-2 appears to be responsible for utilization of both fMet-tRNA^{Met} and Met-tRNA^{Met} in initiation. If this is the case, then disruption of the P. aeruginosa chromosomal infB gene is expected to be lethal. To this test this possibility, we attempted to replace the chromosomal infB gene with a disrupted copy by homologous recombination in P. aeruginosa with or without the pUCP26 plasmid. The two strains were transformed with the suicide vector pEX18APΔKin/F:Gm, and transformants containing the suicide vector in the chromosome were identified by selecting for carbenicillin and gentamicin resistance. Resolution of the plasmid was achieved by subjecting the merodiploid strain to sucrose counterselection in the presence of gentamicin.

Gentamicin- and sucrose-resistant transformants were obtained within 24 h of incubation at 37 °C. Replacement of the wild type infB gene with the mutant copy in several gentamicin- and sucrose-resistant transformants was assessed by PCR
P. aeruginosa Strains & fmt^* & fmt & fmt & fmt^* & fmt & fmt & fmt

pUCP26 & + & + & + & |
MTF & + & + & + & |
IF-2 & + & + & + & |

Base Treatment & + & |

\[\text{Fig. 3. Analysis of the formylation status of the initiator tRNA in wild type and mutant MTF } P. \text{ aeruginosa strains overproducing IF-2 or MTF.} \]

Total RNA was separated on a 6.5% polyacrylamide gel containing 8M urea at 4°C using 100 mM sodium acetate buffer, pH 5.0, and transferred onto Nytran membrane. The various forms of the initiator tRNA were detected with 5'-CGGGTTATGAGCCCG-3', which is complementary to nucleotides 28–42 of the anticodon stem loop.

\[\text{Fig. 4. Verification of the disruption of the } P. \text{ aeruginosa chromosomal infB gene by PCR analysis.} \]

Disruption of the chromosomal infB gene in P. aeruginosa without or with pUCPLIF2 was carried out using pEX18AP\(\Delta\)KinIF2::Gm^R. Replacement of the wild type gene with the mutant copy was assessed by PCR using the primer combinations P1/P2, P1/P3, and P1/P4. The location of the primers and the size of the fragments expected for the wild type and mutant infB genes are shown in the top panel. WT, wild type.

Analysis as shown in Fig. 4. Two products of ~2 and 1 kilobase pair, corresponding to the expected size of the wild type infB gene and the infB::Gm^R fragment, respectively, were observed in the gentamycin- and sucrose-resistant transformants with (left panel, lanes 6 and 7) and without (lanes 3–5) the pUCPLIF2 plasmid when primers P1 and P2, which are complementary to a segment of the 3'- and 5'-ends of the infB gene, respectively, were used. The 1-kilobase pair fragment but not the 2-kilobase pair fragment was observed in the wild type P. aeruginosa strain without (lane 1) and with the pUCPLIF2 plasmid (lane 2). A 1.6-kilobase pair fragment, corresponding to the expected size of the mutant infB::Gm^R gene, was de-
detected in the transformants with (middle panel, lanes 6 and 7) and without (lanes 4 and 5) pUCPLIF2 using P3, which is complementary to the 5' end of the GmR gene and P1. As expected this fragment was not observed in the wild type P. aeruginosa strain without (lane 2) and with (lane 3) pUCPLIF2 but was detected when the pEX18APΔKinB::GmR plasmid was used as the template (lane 1). These results indicated that the mutant copy of the infB gene is present in the chromosome of transformants with and without the pUCPLIF2 plasmid. To ascertain whether the mutant infB::GmR gene was inserted into the infB locus, PCR analysis was conducted with P1 and P4 that are complementary to a segment of the 3' end of the nusA gene, which is located upstream of the infB gene. A 4-kilobase pair fragment of the expected size was observed in transformants with pUCPLIF2 (right panel, lanes 8 and 9) but not in transformants lacking the plasmid (lanes 4–7) or the wild type strains (lanes 2 and 3) or when the pEX18APΔKinB::GmR plasmid was used as the template (lane 1). A 3.1-kilobase pair fragment, corresponding to the wild type allele, was observed in the wild type strains with and without pUCPLIF2 and in the gentamycin- and sucrose-resistant transformants lacking pUCPLIF2. An unspecific 2.9-kilobase pair fragment was obtained when pEX18APΔKinB::GmR was used as the template (lane 1). This product was not present in any of the transformants, indicating that the pEX18APΔK plasmid had been eliminated by double crossover, which is in agreement with the sucrose-resistant phenotype. This analysis showed that the chromosomal infB gene was replaced in cells with the pUCPLIF2 plasmid but not in those without the plasmid. The location of the mutant IF-2 gene in the gentamycin- and sucrose-resistant transformants lacking pUCPLIF2 was not ascertained. These results demonstrate that the wild type chromosomal infB gene can only be disrupted if the strain harbors a second copy of the gene and suggest that IF-2 is essential for survival of P. aeruginosa. This is consistent with the finding that the plasmid containing the wild type gene is retained by the P. aeruginosa infB mutant strain after numerous rounds of growth in the absence of tetracycline selection for the plasmid but is easily lost from the wild type strain after three rounds. The data support the notion that the P. aeruginosa IF-2 is required for both formylation-dependent and -independent protein initiation in P. aeruginosa. These results also exclude the involvement of an unidentified initiation factor in formylation-independent initiation of protein synthesis.

Overexpression of the E. coli IF-2 Is Unable to Compensate for the Loss of Formylation in P. aeruginosa—Complementation of the P. aeruginosa MTF mutant strain was used to investigate whether the E. coli IF-2, like the P. aeruginosa IF-2, has the capacity to facilitate formylation-independent protein initiation (Fig. 5). The P. aeruginosa MTF mutant strain harboring pUCP26 with the coding sequence of the P. aeruginosa IF-2 gene grew more slowly (sector 6) than the wild type strain harboring pUCP26 without (sector 1) and with the E. coli IF-2 (sector 2) or the P. aeruginosa IF-2 gene (sector 3). In contrast, very little growth was observed for the MTF mutant strain harboring pUCP26 alone (sector 4) or pUCP26 carrying the E. coli IF-2 gene (sector 5). Thus, it appears that the E. coli IF-2, unlike the P. aeruginosa IF-2, is unable to facilitate formylation-independent protein initiation. This is consistent with a previous report showing that overexpression of the E. coli IF-2 did not rescue the severe growth defect of an E. coli MTF mutant strain (23). Taken altogether, the data suggest that IF-2 is principally responsible for utilization of unformylated Met-tRNA^{Met} in protein initiation in P. aeruginosa.

Comparative Modeling of the tRNA-Binding Domains of the P. aeruginosa and E. coli IF-2s—To understand why the P. aeruginosa IF-2 but not the E. coli IF-2 is able to facilitate utilization of Met-tRNA^{Met} in protein initiation, the structure of the tRNA-binding domain of the two proteins was evaluated. The tRNA-binding site of IF-2 has been previously been located in the C-terminal subdomain C2 of the B. stearothermophilus IF-2 (33, 34), and the solution structure of this domain as a six-stranded β barrel has been determined (Protein Data Bank code 1DN1) (27). The C2 subdomains of the E. coli and P. aeruginosa IF-2s align with the C2 subdomain of the B. stearothermophilus IF-2 with 45% identity and no gaps; between the E. coli and P. aeruginosa IF-2s, the sequences of the C2 subdomains are 76% identical (Fig. 6A). The structure 1DN1 served as a template for structural models of the E. coli and P. aeruginosa IF-2 C2 subdomains. Both models showed rapid convergence to energy minimum.

The tRNA-binding site of the IF-2 C2 subdomain has been deduced by structural superimposition (1.36 Å root mean square deviation) of the IF-2 C2 subdomain onto the tRNA-binding domain II of the T. aquaticus EF-Tu (28). Sequence alignments (Fig. 6B) derived from the structural superimposition showed 18% identity between the T. aquaticus EF-Tu and the E. coli IF-2 C2 subdomain with many identities and similarities at the tRNA-protein interface. The crystal structure of the T. aquaticus EF-Tu (Protein Data Bank code 1B23) includes bound cysteinyl-tRNA^{Cys} (Fig. 7a), and this allowed the aminoacyl-tRNA to be merged into the structure of the E. coli and P. aeruginosa IF-2 C2 subdomains. Fig. 7b shows the surface charge distribution relative to the proposed tRNA-binding site for the structural model of the P. aeruginosa IF-2 C2 subdomain. In the E. coli IF-2 C2 subdomain (Fig. 7c), a more extensive region of positive charge density attributed to Arg-847 is found adjacent to the amino group of the aminoacyl-tRNA (see Fig. 8b). This suggests that formylation of the amino group is not necessary or energetically unfavorable to form the complex. In the P. aeruginosa IF-2 C2 subdomain, the positive contour due to the equivalent Arg-798 is not quite so extensive, and this may diminish electrostatic repulsion so that the unformylated aminoacyl-tRNA can be tolerated (see Fig. 8c). EF-Tu has neutral Met-272 in the equivalent location (Fig. 6B).

Although the sequences of the E. coli and P. aeruginosa IF-2 C2 subdomains are identical in the immediate tRNA-3'-aminoacyl contact surface, there are subtle differences in the next layer of amino acids that may account for the difference in surface electrostatics. In the E. coli IF-2 C2 subdomain, Glu-854 appears to form an ion pair with Lys-823 (Fig. 8b, blue side
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FIG. 6. A, aligned sequences of the IF-2 C2 subdomains. BACST, the B. stearothermophilus IF-2 sequence contained in the structure Protein Data Bank code 1D1N was used as a template to construct models. E. coli, E. coli; PA, P. aeruginosa. Asterisks indicate residues identical in all three sequences (45%), and caret indicates residues identical between the E. coli and P. aeruginosa IF-2 C2 subdomains. The putative ion-paired residues Glu-805 and Lys-823 in the E. coli IF-2 C2 subdomain are underlined as are the corresponding Glu-805 and His-774 in the P. aeruginosa IF-2 C2 subdomain. Residues forming the direct contact with the aminoaoyl group and A76 are marked aaa, and residues proximal to the 5' phosphate are marked 5'p. B, structural alignment of the T. aquaticus IF-Tu domain II (Protein Data Bank code 1B23) onto the E. coli IF-2 C2 subdomain. This alignment showed 18% identity between EF-Tu and the E. coli IF-2 C2 subdomain. Arg-847, which appears to discriminate formylated proteins on growth of the MTF mutant strain expressing the wild type IF-2. Expression of the wild type IF-2 did not facilitate growth of the MTB mutant strain expressing the wild type IF-2, the H774K mutant (red side chains). In the P. aeruginosa IF-2 C2 subdomain, the equivalent Glu-805 is juxtaposed with His-774, which is not well placed for ion pair formation. This appears to make Glu-805 available for electrostatic interaction with Arg-798 (Fig. 8a, blue side chains). The in silico mutations E. coli K823H and P. aeruginosa H774K (Fig. 8, red side chains) appear to confirm this. The positive potential around the aminoaoyl group is substantially dissipated in E. coli K823H (Fig. 7d). The converse mutation, P. aeruginosa H774K, which should reduce Glu-805 from interaction with Arg-798 (Fig. 8a), results in an expanded region of positive potential adjacent to the tRNA-3'-aminoacyl (Fig. 7c).

The Reduced Positive Charge Potential of the Cleft Involved in Binding the Amino End of the Amino Acid Attached to the tRNA Allows the P. aeruginosa IF-2 to Facilitate Formylation-independent Protein Initiation—We investigated whether increasing the positive charge potential of the tRNA-binding site of the P. aeruginosa IF-2 by mutating His-774 to Lys and decreasing the charge in the E. coli IF-2 by changing Lys-823 to His will affect their ability to facilitate utilization of unfumyalted Met-tRNA\(^{\text{Met}}\) in protein initiation. This was evaluated by assessing the effect of overexpression of the P. aeruginosa H774K (Fig. 9A) and the E. coli K823H (Fig. 9B) mutant proteins on growth of the P. aeruginosa MTB mutant strain on minimal medium. The MTB mutant strain with the pUC26 vector alone did not grow over a 36-h period, whereas significant growth was observed for the mutant strain with the pUC26 vector harboring the wild type P. aeruginosa IF-2 gene (Fig. 9A and B). During the first 27 h of incubation of the MTB mutant strain with the pUC26 vector containing the H774K IF-2 gene very little growth was observed (Fig. 9A). However, some growth of the MTB mutant strain expressing the H774K IF-2 mutant protein was observed within 36 h (Fig. 9A). This growth was significantly slower than the strain expressing the wild type IF-2. Expression of the wild type E. coli IF-2 did not facilitate growth of the MTB mutant strain incubated for 48 h (Fig. 9B). However, the strain grew when the E. coli K823H IF-2 mutant protein was expressed (Fig. 9B). Growth of the MTB mutant strain expressing the E. coli K823H IF-2 mutant protein was slower than that of the strain expressing the wild type P. aeruginosa IF-2. These results indicate
that changing His-774 to Lys dramatically reduced the ability of the \textit{P. aeruginosa} IF-2 to facilitate formylation-independent protein initiation, whereas mutating Lys-823 to His caused the \textit{E. coli} IF-2 to facilitate utilization of Met-tRNA\textsuperscript{Met} in protein initiation. Taken together, the data suggest that the reduced positive charge potential of the cleft in the \textit{P. aeruginosa} IF-2 is a key determinant allowing the \textit{P. aeruginosa} IF-2 to bind both initiator methionyl-tRNA and formyl-methionyl-tRNA and facilitate their use in protein initiation in \textit{P. aeruginosa}. Furthermore the results suggest that in the \textit{E. coli} IF-2 the positive charge potential of the cleft prevents the protein from binding the Met-tRNA\textsuperscript{Met}, excluding participation of the unformylated tRNA in initiation. This may explain why bacteria such as \textit{E. coli} are dependent on formylation for initiation of protein synthesis. It may, therefore, be possible to use this structural characteristic and the \textit{P. aeruginosa} formylation-defective strain as a tool to identify eubacterial IF-2s with the ability to facilitate protein initiation with both the formylated and unformylated species of Met-tRNA\textsuperscript{Met} and to predict whether a bacterium is capable of both formylation-dependent and -independent protein initiation or relies primarily on formylation-dependent initiation.

The Surface Charge Potential of the Cleft of the Human and Yeast Mitochondrial IF-2 C2 Subdomains Involved in Binding the Amino End of the Aminoacyl-tRNA Is Nearly Neutral—Eubacterial IF-2s consist of an N-terminal region, a G-domain that binds GTP, and a C-terminal region involved in binding tRNA. The G-domain and C-terminal region are conserved among the eubacterial and mitochondrial IF-2 proteins. The \textit{S. cerevisiae} and mammalian mitochondrial IF-2s have been shown to interact well with the \textit{E. coli} Met-tRNA\textsuperscript{Met} in vitro (35, 36), and like the \textit{P. aeruginosa} IF-2 both proteins were able to allow formylation-independent mitochondrial protein initiation in a \textit{S. cerevisiae} strain with an inactivated mitochondrial MTF gene (18). Thus, we investigated by structural modeling whether the cleft in the two mitochondrial IF-2s has a positive charge potential comparable to the \textit{P. aeruginosa} IF-2. Fig. 10 indicates that the surface charge of the yeast and human mitochondrial IF-2 C2 subdomains is near neutral adjacent to the aminocyl group. This characteristic could explain why these proteins are able to facilitate the use of the unformylated Met-tRNA\textsuperscript{Met} in mitochondrial protein initiation.

In formylation-dependent bacteria such as \textit{E. coli}, the fMet moiety of fMet-tRNA\textsuperscript{fMet} is the major determinant that allows IF-2 to distinguish the initiator tRNA from elongator tRNAs and Met-tRNA\textsuperscript{Met}. Since the \textit{P. aeruginosa} IF-2 has the capac-

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**FIG. 9.** Analysis of the ability of the H774K \textit{P. aeruginosa} and K823H \textit{E. coli} IF-2 mutants to compensate for the loss of formylation in the \textit{P. aeruginosa} MTF mutant strain. \textit{a}, the H774K \textit{P. aeruginosa} IF-2 mutant is unable to participate in formylation-independent protein initiation. The mutant MTF \textit{P. aeruginosa} strains harboring the pUCP26 vector without and with the \textit{P. aeruginosa} wild type or mutant \textit{infB} gene were streaked on M9 glucose medium containing 60 \mu g/ml tetracycline and incubated at 37 °C for the times indicated. \textit{b}, the K823H \textit{E. coli} IF-2 mutant facilitates formylation-independent protein initiation. The \textit{P. aeruginosa} MTF mutant strains harboring pUCP26 with the wild type \textit{P. aeruginosa} or \textit{E. coli} \textit{infB} gene or the \textit{E. coli} mutant \textit{infB} gene were streaked on M9 glucose medium containing 60 \mu g/ml tetracycline and incubated at 37 °C for 48 h.

**FIG. 10.** Structural models of the yeast and human IF-2s and the surface charge potential of the cleft involved in binding the amino end of the aminocyl group attached to the tRNA. \textit{a}, the \textit{S. cerevisiae} mitochondrial IF-2 C2 subdomain, modeled onto the \textit{B. Stearothermophilus} template 1D1N, with Cys-tRNACys merged from EF-Tu 1B23. \textit{b}, the human mitochondrial IF-2 C2 subdomain; the model was derived as for yeast.
ity to facilitate utilization of both Met-tRNAfMet and fMet-tRNAfMet in protein initiation, this raises the question as to how the *P. aeruginosa* IF-2 discriminates between initiator and elongator tRNAs. The structural model of the *P. aeruginosa* IF-2 C2 subdomain with Cys-tRNA*CyS* indicates that the 5′-phosphate (Fig. 7b, large arrow) is in close proximity to a region of high negative charge. This could potentially result in a steric clash that would most likely destabilize the interaction between IF-2 and the tRNA. Therefore, it is unlikely that the 5′-phosphate group of the initiator tRNA is near this region in IF-2. A significant difference between the eubacterial initiator and elongator tRNAs is the absence of a base pair between nucleotides 1 and 72 in the acceptor stem of the initiator tRNA. This is due to the presence of a C at position 1 and an A at position 72. Thus, we surmise that the C1 × A72 mismatch allows the *P. aeruginosa* IF-2 to open up the acceptor stem of the initiator tRNA to correctly position the 5′-phosphate group within the positive charge region containing Lys in the *P. aeruginosa* IF-2 and Arg in the *E. coli* protein (Figs. 6 and 7b, arrowhead). Accordingly we propose that the *P. aeruginosa* IF-2 discriminates between elongator and initiator tRNAs based on the correct interaction with the 5′-phosphate group. This explanation is consistent with the finding that a minor determinant in the *E. coli* initiator tRNA recognized by the *E. coli* IF-2 is the C1 × A72 mismatch (26).

We have shown that the *P. aeruginosa* IF-2 is responsible for both formylation-dependent and -independent protein initiation in *P. aeruginosa*. This represents the first demonstration of a eubacterial IF-2 with an inherent capacity to facilitate the use of Met-tRNA*fMet* in protein initiation, discounting the use of Met-tRNA*fMet* in initiation of protein synthesis. The data suggest that this property is not unique to the *P. aeruginosa* IF-2 since the *E. coli* IF-2 was made to participate in formylation-independent protein initiation by a single modification that reproduces the charge distribution in the *P. aeruginosa* IF-2. This opens up the possibility that other eubacterial IF-2 proteins may have the capabilities to allow utilization of Met-tRNA*fMet* in protein initiation especially for those bacteria that have been shown to have the ability to carry out formylation-independent initiation. The availability of a large number of eubacterial infB sequences combined with the strategy used in this study will facilitate investigation of this question. These and other studies will be needed to help understand why some bacteria have the ability to initiate protein synthesis with both Met-tRNA*fMet* and fMet-tRNA*fMet* while others rely only on formylation.
The *Pseudomonas aeruginosa* Initiation Factor IF-2 Is Responsible for Formylation-independent Protein Initiation in *P. aeruginosa*

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*J. Biol. Chem. 2004, 279:52262-52269.*
doi: 10.1074/jbc.M408086200 originally published online September 22, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M408086200

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