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Elongation Factor Tu Is Methylated in Response to Nutrient Deprivation in *Escherichia coli*

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It has been shown previously that starvation of a mid-logarithmic-phase culture of *Escherichia coli* B/r for an essential nutrient results in the methylation of a membrane-associated protein (P-43) (C. C. Young and R. W. Bernlohr, J. Bacteriol. 172:5147–5153, 1990). In this communication, the purification of P-43 and sequence analysis of cyanogen bromide-generated peptide fragments identified P-43 as elongation factor Tu (EF-Tu). This was confirmed by the ability of anti-EF-Tu antibody to precipitate P-43. We propose that the nutrient-dependent methylation of EF-Tu may be involved in the regulation of growth, possibly as a principal component of an unidentified signal transduction pathway in bacteria.

Bacterial cells respond to environmental change through the differential expression of families of proteins that are required for survival during periods of stress. During starvation, these proteins allow the cell to utilize nutrients more efficiently and confer upon the cell a stress-resistant phenotype. The degradation of intracellular proteins and RNA presumably provides the necessary precursors for the cell to synthesize approximately 40 proteins that are regulated in response to starvation (20). Of these, approximately 13 proteins are common to nitrogen, carbon, and phosphate starvation and have been designated Pex proteins. Many of the 40 proteins induced by starvation are also induced during heat shock and oxidative stress, indicating that a single response may have evolved that can be activated by various factors. This is supported by studies showing that starvation can confer a strong cross-protection against heat shock, osmotic shock, oxidative stress, and antibiotics (for a review, see reference 20).

Our laboratory has previously reported that a membrane-associated protein in *Escherichia coli* (25), *Bacillus subtilis* (9), and *Bacillus licheniformis* (2) is differentially methylated in response to the availability of nutrients in the medium. The *Bacillus* protein (P-40) has a molecular mass of approximately 40 kDa and is methylated when nutrients are added to a starving culture. The methyl groups on P-40 turn over with a half-life of 10 to 15 min. The protein is not involved with chemotaxis, and the demethylation process is defective in some stage 0 mutants that are unable to sporulate and are, therefore, constitutive for growth (9). In contrast, the *E. coli* protein (P-43) is methylated in response to depletion of nutrients in the medium (25). The protein is not involved in chemotaxis and is labeled on lysine residues. When *E. coli* is starved for an essential nutrient, P-43 is methylated with a doubling time of approximately 5 min, whereas introduction of the missing nutrient to the medium results in an immediate cessation of P-43 methylation followed by a slower demethylation process.

This communication describes the purification and identification of the P-43 molecule as elongation factor Tu (EF-Tu) and discusses the possible implications for bacterial nutrient sensing.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. Cultures of *E. coli* B/r were grown at 37°C as described previously (25), except that 100 mM morpholinepropanesulfonate (MOPS) buffer (pH 7.5) was used instead of potassium phosphate buffer in the growth medium.

Preparation of crude extracts of P-43. P-43 was labeled and extracted from membrane fragments by the procedure described by Young and Bernlohr (25), except that 250 μCi (60 nM) of [*methyl*-^{3}H]methionine (80 Ci/mmol) was added to the culture instead of 25 μCi.

Purification of P-43. Elution of protein from all columns was monitored (optical density at 280 nm) while 5-μl samples were removed from each fraction to determine the radioactivity elution profile. After each purification step, fractions containing radioactivity corresponding to P-43 were pooled, and total protein was determined by the method of Lowry et al. (18). Approximately 15 μg of each pooled protein sample was saved for polyacrylamide gel electrophoresis (PAGE) and autoradiography in order to confirm the presence of P-43 in the sample. Membrane extracts were applied to a Sephacryl S-300 molecular sieving column (1.5 by 40 cm) (Pharmacia LKB, Uppsala, Sweden) operated at 15 ml/h with a buffer containing 10 mM Tris-HCl (pH 8.1) and 1 mM phenylmethylsulfonyl fluoride (PMSF), and 4-ml fractions were collected. Radioactive protein corresponding to P-43 (fractions 11 to 16) were pooled and applied to a heparin agarose column (1 by 10 cm) (Sigma, St. Louis, Mo.) equilibrated and washed with 10 mM Tris-HCl (pH 8.1). Fractions (2 ml) were collected at a flow rate of 0.5 ml/min. Under these conditions, no radioactivity eluted in the wash. The addition of 25 mM Tris-HCl (pH 8.1) containing 1 mM PMSF resulted in the immediate elution of P-43. The radioactive protein mixture that eluted from the heparin agarose column was applied to a DEAE-cellulose column (1 by 10 cm) (Sigma) equilibrated with 10 mM Tris-HCl (pH 8.1) and 1 mM PMSF. All of the radioactive material bound to the column under these conditions. Radioactive protein was eluted from the column by using a 50 ml, 0 to 0.25 M NaCl linear gradient in 10 mM Tris-HCl (pH 8.1) containing 1 mM PMSF. The radioactive protein that eluted from this column was concentrated and washed in a Centricon 10 microconcentrator (Amicon, Danvers, Mass.) before separation by two-dimensional (2D) gel electrophoresis, performed as described by Hall and Killian (11). Protein purified through 2D

gel electrophoresis was tested for purity by reverse-phase high-pressure liquid chromatography (HPLC) by using a Delta-pak C_{18} column (Waters, Milford, Mass.). Radioactive protein was removed from 2D polyacrylamide gel slices and loaded onto the column by on-line gel elution. A gradient of 10 to 90% acetonitrile containing 0.1% trifluoroacetic acid was used to elute protein from the column, and this was monitored both by spectrophotometry (optical density at 280 nm) and by scintillation counting of all fractions.

Antibody precipitation of P-43. Antibody precipitation experiments were performed as described by Coussens et al. (6), with slight modifications. Briefly, membrane proteins from a 50-ml culture of *E. coli* B/r were labeled and isolated as described previously (25). Twenty-five micrograms of membrane proteins was diluted to a final volume of 100 μl in phosphate-buffered saline and incubated in the presence of goat anti-EF-Tu antibody with gentle shaking. After a 60-min incubation, 0.1 ml of anti-goat immunoglobulin G (IgG) bound to agarose beads (Sigma) was added, and the mixture was incubated for an additional 60 min. Antibody-protein complexes were precipitated by centrifugation and separated by PAGE. Polyacrylamide gels were stained with Coomassie blue to visualize total protein, followed by autoradiography to observe radioactive protein. As a control, a separate precipitation reaction was carried out exactly as the first, except that no anti-EF-Tu antibody was added to the extract.

Other methods. Cyanogen bromide digestion was performed by the method of Goossens et al. (10). Electroblotting onto Immobilon polyvinyl difluoride transfer membranes (Millipore Corp., Bedford, Mass.) was performed as described by Kyhse-Anderson et al. (16). Isotopic methods and instruments used were described previously (2).

RESULTS

Purification of P-43. In an effort to identify the methylated P-43 protein, labeled membrane extracts were prepared from a 50-ml culture of *E. coli* B/r that had been starved for ammonia (25). This extract containing ^{3}H-methylated proteins was combined with a second, unlabeled membrane extract prepared from a 2-liter culture of *E. coli* B/r. The resulting 10-ml membrane extract contained approximately 400 mg of protein and 10^8 cpm, of which approximately 4% was due to P-43 methylation, as determined by densitometry of autoradiographs after PAGE (Fig. 1B, lane 1). Most of the remaining radioactivity was due to cyclopropane fatty acids formed during the starvation period that migrated on polyacrylamide gels at a molecular mass of <10,000 Da (25) (Fig. 1B, lane 1). Some minor radioactive bands have been observed when gels were overexposed; however, these bands were not reproducible and the label in these proteins was not dependent upon nutrient availability (25).

The ^{3}H-labeled protein (P-43) in the membrane extract was purified to homogeneity by using a combination of Sephacryl S-300, heparin agarose, and DEAE-cellulose column chromatography followed by 2D gel electrophoresis (Table 1). Fractions eluting from the Sephacryl S-300 molecular sieving column that contained radioactive protein corresponding to a molecular mass of 43,000 Da were pooled. A portion of this pooled protein was separated on a sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gel to prove that the radioactivity in the pooled fraction comigrated with P-43 (Fig. 1B, lane 2), whereas the rest of the protein was separated on a heparin agarose column. All of the radioactivity bound to the heparin agarose column in 10 mM Tris-HCl (pH 8.1). The addition of 25 mM Tris-HCl (pH 8.1)–1 mM PMSF resulted in the immediate elution of >90% of the radioactivity that migrated at a molecular mass of 43,000 Da on polyacrylamide gels (Fig. 1B, lane 3), resulting in the protein profile shown in Fig. 1A, lane 3. The remaining radioactivity, including >99% of the low-molecular-weight radioactivity, eluted in a 1 M Tris-HCl (pH 8.1)–1 mM PMSF wash. The protein that eluted from the heparin agarose column was applied to a DEAE-cellulose column through which a 0 to 0.25 M NaCl gradient was passed. All of the radioactivity eluted from this column in a single fraction at approximately 0.18 M NaCl. Autoradiography of polyacrylamide gels containing protein purified by DEAE-cellulose chromatography showed that a significant amount of radioactivity was once again migrating at an apparent molecular mass of <10,000 Da (Fig. 1B, lane 4). Because all of the radioactivity after the heparin agarose column had been shown to migrate at a molecular weight corresponding to that of P-43, we attributed the low-molecular-weight radioactivity to either a loss of methyl groups from P-43 or breakdown of the protein itself. Because any further chromatographic purification procedures resulted in the conversion of >95% of the radioactivity to the lower-molecular-weight form, pooled fractions from the DEAE-cellulose column were immediately concentrated, washed to remove salt, and separated by 2D gel electrophoresis (11). Proteins were visualized with Coomassie blue, and gels were autoradiographed to determine the location of radioactivity. Autoradiographs of these gels revealed a single radioactive protein at a molecular mass of 43,000 Da with an isoelectric point of 6.1. The radioactive protein was cut from 2D gels and applied directly to a C_{18} reverse-phase column. Protein from this column eluted as a major protein component with a shoulder (Fig. 2). Absorption analysis of the shoulder region did not display the classical protein absorption spectrum, and visual inspection of the corresponding sample showed a distinct blue coloration. The major component

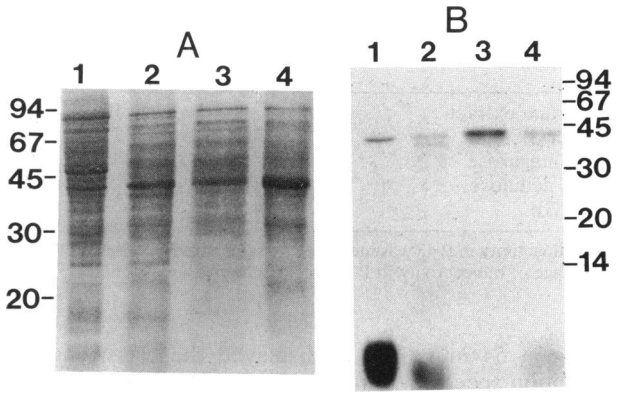

FIG. 1. PAGE of membrane proteins after each column purification procedure. An equal amount of protein from each column purification step was loaded onto an SDS–12.5% polyacrylamide gel to verify the presence of P-43 throughout the purification procedure. (A) Coomassie blue-stained gel of the total membrane extract (lane 1) and of protein remaining after Sephacryl S-300 column chromatography (lane 2), after heparin agarose column chromatography (lane 3), and after DEAE-cellulose column chromatography (lane 4); (B) autoradiograph of the same gel shown in panel A, indicating the presence of labeled P-43 in the membrane extract (lane 1) and after Sephacryl S-300 column chromatography (lane 2), after heparin agarose column chromatography (lane 3), and after DEAE-cellulose column chromatography (lane 4).

TABLE 1. Summary of P-43 purification from membrane extracts of *E. coli* B/r

Method	Protein concn (mg/ml)	Total vol (ml)	Total protein (mg)	Radioactivity[a] in P-43 (cpm)	Radioactivity/ total protein	Purification (fold)
Membrane extract	40	10	400	4.1×10^6	1.0×10^4	
Sephacryl S-300	3.4	32	110	4.0×10^6	3.6×10^4	3.6
Heparin agarose	1.1	50	55	3.5×10^6	6.3×10^4	6
DEAE-cellulose	0.5	6	3	8.8×10^5	2.9×10^5	28
2D PAGE			0.5	7.5×10^5	1.5×10^6	145

[a] Radioactivity in P-43 was measured by scintillation counting of the whole membrane extract and determination of the percentage of radioactivity in P-43. The percentage of radioactivity in P-43 was determined by quantitating the intensity of bands on autoradiographs by densitometry.

eluting at 54 min (Fig. 2) displayed the classical protein absorption spectrum, with maximal absorption occurring at 227 and 274 nm. This fraction also contained >80% of the radioactivity. From this we concluded that the shoulder fraction eluted slightly earlier because of bound Coomassie blue remaining from the visualization of the 2D gel even after extensive destaining and that the protein from 2D gel slices was homogeneous.

Identification of P-43. Protein purified by the above procedure was digested with cyanogen bromide within 2D gel slices, and the fragments were separated on an SDS–12.5% polyacrylamide gel. Peptide fragments were blotted onto polyvinyl difluoride transfer membranes (16), and the membranes were stained with Coomassie blue. This allowed for the visualization of two peptide bands that migrated at an apparent molecular mass near 12,000 Da. These peptides were sequenced at the Wistar Institute Protein Microchemistry Core Facility. The resulting sequences obtained were FRKLLDEGRAGENVGVLLRGIKREXIERG and EVRE LLSQYDFPGDDTPIXXXXAL (X denotes unidentified amino acid). A search of the National Biomedical Research Foundation protein sequence data base revealed that the sequences matched perfectly to amino acid positions 262 to 290 and 153 to 176, respectively, of *E. coli* EF-Tu. On the basis of this sequence data, we tentatively identified P-43 as EF-Tu.

To confirm that P-43 was indeed EF-Tu, membrane extracts from labeled *E. coli* B/r starved for ammonia were precipitated with goat anti-EF-Tu antibody by the method of Coussens et al. (6), and the precipitated proteins were separated by electrophoresis on an SDS–12.5% polyacrylamide gel. The results showed that a protein precipitated when membrane extracts were incubated in the presence of anti-EF-Tu antibody (Fig. 3, lane 3) but not in a control without anti-EF-Tu antibody (Fig. 3, lane 4) and that this protein comigrated with purified EF-Tu (Fig. 3, lane 2). Autoradiography of the same gel showed that the precipitated protein was highly labeled (Fig. 3, lane 7) and that the label comigrated with P-43 (Fig. 3, lane 5). In addition to P-43, a number of protein bands were observed in the stained polyacrylamide gel both in the anti-EF-Tu precipitation reaction (lane 3) and in the control (lane 4). These same bands were observed upon electrophoresis of anti-goat IgG-bound agarose beads or anti-EF-Tu antibody and therefore appear to be due to the presence of IgG in the precipitation reaction. On the basis of these results, we concluded that the methylated protein that we termed P-43 is actually a membrane-associated form of EF-Tu.

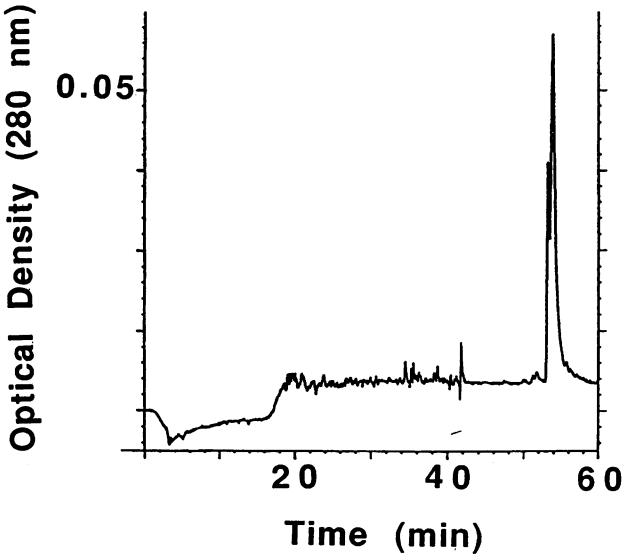

FIG. 2. Reverse-phase C_{18} HPLC elution profile of purified protein excised from 2D gels. A single major component containing a shoulder eluted in approximately 76% acetonitrile (54 min) is shown. On the basis of its unique absorption spectra and a distinct blue coloration of the corresponding sample the shoulder region is believed to be a result of Coomassie blue-bound P-43 (See Results).

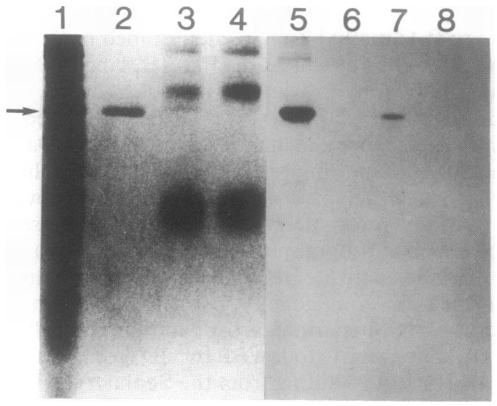

FIG. 3. Precipitation of P-43 with anti-EF-Tu antibody. Lanes 1 to 4, Coomassie blue-stained SDS-polyacrylamide gel of a membrane extract (lane 1), purified EF-Tu (lane 2), crude extract treated with goat anti-EF-Tu antibody and anti-goat IgG bound to agarose beads (lane 3), and a control containing crude extract and anti-goat IgG bound to agarose beads (lane 4). Lanes 5 to 8, autoradiograph of the same gel. The arrow marks the position of EF-Tu on the polyacrylamide gel.

DISCUSSION

EF-Tu is a multifunctional protein which promotes the binding of aminoacyl-tRNAs to the ribosome. This requires the interaction of EF-Tu with a variety of factors, including aminoacyl-tRNA, ribosomes, GTP, GDP, and elongation factor Ts. In addition, EF-Tu also interacts with phage-encoded proteins to form an integral part of the Qβ replicase (3), and it may interact with one or more proteins in the transcriptional apparatus to act as a positive regulator of RNA synthesis (11, 22). EF-Tu was previously shown to be methylated on lysine 56 (17). Net amounts of monomethyl lysine at this position were converted to dimethyl lysine during the stationary phase of growth (7), but no hypothesis for the role of methylation was advanced. In addition, EF-Tu has been shown to be membrane associated in *E. coli* (17). This has been proposed to be of structural significance because of similar physical properties between EF-Tu and actinlike proteins (17).

This report identifies P-43 as EF-Tu. Therefore, the methylation of EF-Tu is controlled by the availability of carbon, nitrogen, and phosphate sources in the external medium. This is significant because it indicates that EF-Tu may play an important regulatory role in cell growth and in the bacterial response to nutrient deprivation. Our current hypothesis is that the membrane location of EF-Tu would allow it to interact with one or more unknown nutrient receptor or sensing proteins that would control the state of EF-Tu methylation. Depletion of nutrient in the medium would result in the hypermethylation of EF-Tu and its release from the membrane, allowing EF-Tu to carry out an intracellular regulatory function. On the basis of the known characteristics of EF-Tu, this regulatory function could be twofold.

First, because EF-Tu promotes the binding of aminoacyl-tRNA to the ribosome, it is uniquely positioned to act in a translational control step that could be responsible for shutting down most vegetative protein synthesis during starvation while allowing the synthesis of starvation-induced proteins to continue. Examples of translational control involving the preferential translation of specific mRNAs include (i) the coordination of rRNA and ribosomal protein synthesis in *E. coli* and yeast, (ii) development in *Dictyostelium* spp., (iii) some virus-host interactions, and (iv) the heat shock response in *Drosophila* spp. (1; reviewed in reference 20). It has also been proposed recently that the ribosome acts as the primary sensor during heat and cold shock in *E. coli* by controlling the rate of translation of heat shock proteins (23). X-ray crystallography of GDP-bound EF-Tu has shown that GTP binding occurs in a highly structured domain of the molecule extending from Ile 60 to Glu 240, referred to as the tight domain (21). The proximity of Lys 56 to this binding domain suggests that the methylation of this residue may affect the binding and/or hydrolysis of GTP. This is supported by experiments that indicate that the methylation of EF-Tu may act to slow the rate of GTP hydrolysis in vitro (24). Secondly, EF-Tu has been shown to act as a transcriptional activator in the presence of RNA polymerase and the appropriate sigma factor (12, 22), and it may therefore be capable of regulating the synthesis of proteins which respond to stress at the transcriptional level. However, the effect of methylation on the ability of EF-Tu to stimulate transcription in vitro has not been tested.

On the basis of the similarities between the *E. coli* P-43 and *B. subtilis* P-40 molecules, we have also tentatively identified P-40 as the *B. subtilis* EF-Tu, and experiments are now under way to test this hypothesis. It is interesting to point out that GTP-GDP pools have long been implicated as possible signals for sporulation in *Bacillus* spp. (8). The fact that EF-Tu is a GTP-GDP binding protein, together with our previous results indicating that P-40 showed altered methylation patterns in some *spo0* mutants (9), may implicate *B. subtilis* EF-Tu in the initiation of sporulation.

There are a number of interesting similarities between EF-Tu and other known GTP-binding proteins. Several GTP-binding proteins of eukaryotic organisms, such as Ras and G proteins, transduce environmental signals from the membrane to the interior of the cell in much the same way that we have proposed for the action of EF-Tu. Four principal regions of homology between the amino acid sequences of the H-Ras p21 protein and EF-Tu have been identified (13, 15). In fact, the deduced tertiary structure of EF-Tu by X-ray crystallography has been used as a model to predict the tertiary structure of the α subunit of G proteins (19). Recently, it has been shown that the substitution of threonine for proline 82 in EF-Tu results in autophosphorylation at that site (5), although no phenotype has been described. The substitution of threonine at the homologous site in H-Ras p21 also results in the autophosphorylation of Ras, leading to transformation of the cell. In addition, it has been shown that Ras is carboxymethylated in mammalian cells prior to acylation and transport to the membrane. (4)

The membrane location (14), nutrient-dependent methylation, the ability to act in transcription, the position in the translational apparatus, and similarities to Ras and G proteins all indicate that there may be a second, as yet undetermined role for EF-Tu in the cell. We propose that this function is involved in the ability of the bacterial cell to sense and respond to impending nutrient deprivation, and experiments are now under way to test this hypothesis.

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