In vitro methylation of the elongation factor EF-Tu from *Escherichia coli*

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The in vitro methylation of the elongation factor EF-Tu from *Escherichia coli* was investigated. The methylation of newly synthesized EF-Tu was obtained using λrif^{d}18 DNA as template and *S*-adenosyl [*methyl*-^{3}H]methionine as methyl donor. About 3 mol methyl residues were incorporated for every 10 mol EF-Tu synthesized. Analysis of the nature of the methyl-containing residues by protein hydrolysis followed by paper chromatography showed that both mono- and dimethyllysine were present. The methylation of EF-Tu was also studied separately from its synthesis by using cell-free systems with artificially undermethylated components.

Elongation factor EF-Tu *Protein methylation* *DNA-dependent system* *Ethionine*

1. INTRODUCTION

Many proteins of the translational apparatus are methylated in bacteria [1–4]. One of these proteins is the elongation factor EF-Tu which has been described as being modified in both *Escherichia coli* and *Salmonella typhimurium* [5–7]. *E. coli* EF-Tu showed the presence of mono- and dimethyllysine, 95% of the methyl groups being at a unique site, Lys-56 [5–8].

Interestingly, the equivalent eucaryotic factors (eEF-Tu) from *Mucor racemosus* [9], *Artemia salina* [10] and transformed 3T3 cells [11] have recently been described as being heavily methylated [9–11].

The bacterial elongation factor EF-Tu is essential in protein synthesis [12,13] and also fulfills numerous other activities [14,15]. However, the function of the posttranslational methylation of the factor is completely unknown. Therefore, we have studied the in vitro methylation of EF-Tu from *E. coli* using both a DNA-dependent system and a cell-free system that employs artificially submethylated substrates.

Abbreviations: EF-Tu, elongation factor Tu; AdoMet, *S*-adenosylmethionine; Lys(Me), ε-*N*-monomethyllysine; Lys(Me$_2$), ε-*N*-dimethyllysine; Lys(Me$_3$), ε-*N*-trimethyllysine; M-His, π-methylhistidine; PAGE, polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

E. coli D-10 (*met, rel*) was kindly supplied by Dr D. Hayes and was grown at 37°C in M-9 minimal medium supplemented with 15 μg methionine (control cells), 0.8 μg methionine (low-methionine cells) or with 15 μg ethionine per ml (ethionine cells). Cells grown under these conditions were harvested and bacterial extracts (S-150) from each kind of cell were obtained as in [4].

EF-Tu was purified by affinity chromatography with GDP-Sepharose as described by Jacobson and Rosenbusch [16]. Specific antisera to purified EF-Tu were raised in rabbits as in [17,18]. The in vitro methylation of newly synthesized EF-Tu was measured with a complete system for protein synthesis, using λrif^{d}18 DNA as template as in [17,18]. Incubation was done in the presence of

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[^{35}S]methionine to measure the synthesis of EF-Tu or in the presence of cold methionine and *S*-adenosyl-L-[*methyl*-^{3}H]methionine to determine methylation of the synthesized products [17]. The in vitro methylation of EF-Tu was also determined by using submethylated extracts obtained from *E. coli* grown in the presence of low methionine [19] or ethionine [1,20]. This assay was essentially done as described by Chang and Chang [19] in a final volume of 0.2 ml containing 50 mM NH_4Cl, 5 mM Tris-HCl (pH 7.8), and 1 mM dithiothreitol; 300 μg S-150 submethylated proteins, 135 pmol [*methyl*-^{3}H]AdoMet (74 Ci/mmol) and with 50 μg control S-150 as a source of methylating activity. Unless stated otherwise, incubations were for 25 min at 37°C.

The extent of incorporation of methyl groups into the products of both kinds of system was estimated by determining the hot Cl_3CCOOH-insoluble material or, by immunoprecipitation with EF-Tu antiserum followed by washing of the immunoprecipitates and analysis by PAGE in the presence of SDS as in [17].

The analysis of the methylated amino acids present in EF-Tu was done after the immunoprecipitated protein was separated by PAGE and extracted from the gel. The extracted protein was then hydrolyzed for 24 h in 5.7 N HCl [4,17]. Conditions for paper chromatography of the amino acids obtained have been described [2].

3. RESULTS AND DISCUSSION

3.1. *Methylation of newly synthesized elongation factor EF-Tu*

To study the synthesis and methylation of EF-Tu, we employed a DNA-dependent system [17]. Fig.1 shows a gel pattern of the radioactive products formed in vitro that immunoprecipitated with EF-Tu antiserum. In fig.1A, [^{35}S]methionine was used to label the proteins, whereas in fig.1B, [*methyl*-^{3}H]AdoMet was employed. It was found that one main radioactive protein, of M_r ~43000, was immunoprecipitated with EF-Tu antiserum. Based on an M_r of 43000 and a methionine content of 10 for EF-Tu [21], 3 pmol protein were synthesized in the DNA-dependent in vitro system, in agreement with [22]. Some lower-M_r peaks are also seen (fig.1A,B). These may correspond to degradation of part of the in vitro synthesized EF-Tu or in-

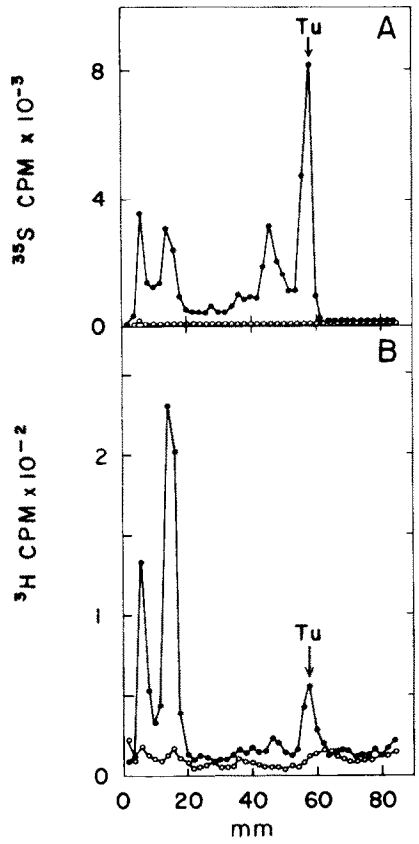

Fig.1. Disc gel electrophoresis of newly synthesized products after precipitation with antiserum to EF-Tu. Reaction mixtures after incubation with either [^{35}S]methionine (A) or [*methyl*-^{3}H]AdoMet (B) were immunoprecipitated with EF-Tu antiserum and the precipitate was washed and solubilized as described in section 2. The solubilized proteins were electrophoresed on SDS-polyacrylamide (10%) gels, sliced an assayed for radioactivity. The incubations were in the presence (●) or absence (○) of λrif^{d}18 DNA. The arrows indicate the position of authentic EF-Tu. Electrophoretic migration was to the left of the figure.

completely translated fragments from the factor [18].

By correcting the specific activity of the radioactive AdoMet added for the dilution with AdoMet formed during the in vitro incubation [17], it was estimated that about 3 mol methyl residues were incorporated for every 10 mol EF-Tu synthesized (i.e., 33%). This value is similar to those reported for the in vivo methylation of EF-Tu in the presence of chloramphenicol [5,6].

3.2. *Methylated amino acids in EF-Tu synthesized in vitro*

It is known that in vitro methylated EF-Tu contains both Lys(Me) and Lys(Me_2) [5,6]. To identify the methylated amino acids in the in vitro synthesized EF-Tu, the methylated protein was hydrolyzed as described in section 2. Fig.2 shows that when the hydrolysis products are separated by paper chromatography, most of the methyl groups correspond to Lys(Me) and Lys(Me_2) in about equal proportions. These results are in close agreement with those reported for the in vivo methylation of EF-Tu [5].

3.3. *In vitro methylation of artificially submethylated EF-Tu*

It is not possible to separate synthesis from methylation of EF-Tu by using the crude DNA-dependent system. Thus, we have used 2 systems in which cells are grown under limiting methylating conditions.

Ethionine is an analog of methionine which is not able to form *S*-adenosylmethionine in bacteria and therefore, undermethylated cellular components can be produced by growing methionine-requiring strains of *E. coli* in minimal media supplemented with ethionine [4,20]. Apparently, these components, in which methionine is replaced by the analog, function normally [20]. The other system consists of growing the same kind of *E. coli* cells (*met, rel*) under limiting methionine concentrations [19] as described in section 2.

Fig.3 shows the time course for the in vitro incorporation of [*methyl*-^{3}H]AdoMet into hot Cl_3CCOOH-insoluble products. Total protein methylation is completely dependent on the undermethylated substrates in both systems. The requirement for the undermethylated substrates indicates that only the artificially obtained products are being methylated under our conditions and perhaps normally, they are almost completely methylated in vivo [5,6].

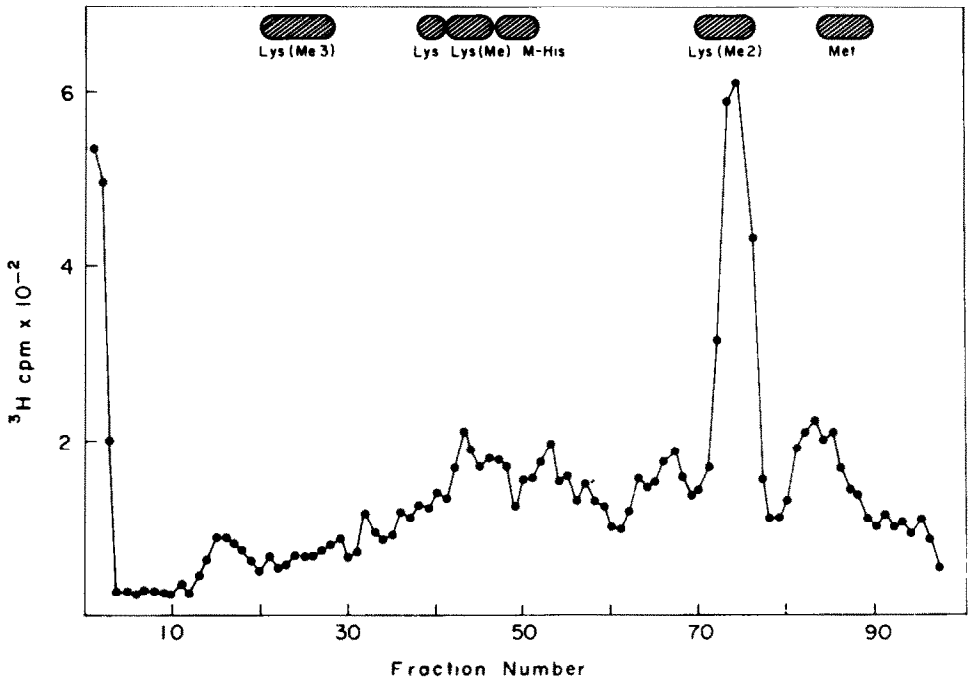

Fig.2. Analysis of the methylated amino acids present in EF-Tu synthesized in vitro using [*methyl*-^{3}H]AdoMet. Paper chromatographic analysis of the protein hydrolysate (described in section 2) was done by applying 50 μl hydrolysate to Whatman no.1 paper together with 10 μg of each of the following standard amino acids: Lys, Met, M-His, LysMe, Lys(Me_2) and Lys(Me_3). The solvent used was pyridine/acetone/3 M NH_4OH (50:30:25, v/v). Conditions for paper chromatography, detection of spots, and determination of radioactivity are as described in section 2.

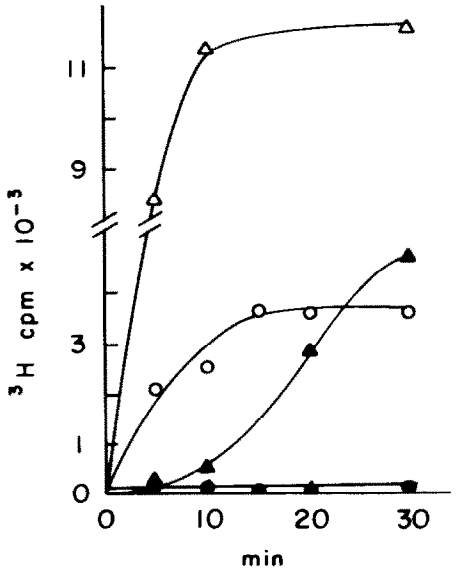

Fig.3. Time course of methylation of total proteins from artificially submethylated extracts. Aliquots from in vitro incubations were removed at various times, and the extent of incorporation of (*methyl*-^{3}H) groups into total proteins (hot Cl_3CCOOH-insoluble) or proteins immunoprecipitated with antiserum to EF-Tu were determined as described in section 2. The values represent total incorporation in the reaction mixture (200 μl). (△—△) Low-methionine extract as substrate; (○—○) ethionine extract; (●—●) control extract; (▲—▲) low-methionine extract after immunoprecipitation.

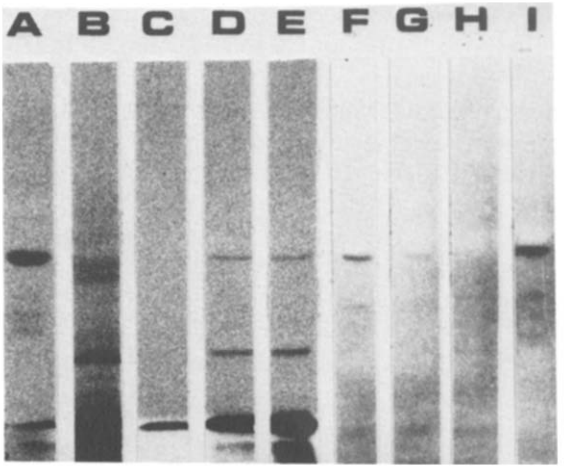

Fig.4. Slab gel electrophoresis of in vitro methylated products after immunoprecipitation with antiserum to EF-Tu. The in vitro incubations in the presence of [*methyl*-^{3}H]AdoMet were as described in fig.3, except they contained 120 μg (C), 370 μg (D) or 600 μg (B,E) ethionine extract or 300 μg low-methionine extract (F,G,H). After incubation, 50 μl of each reaction mixture were immunoprecipitated with antiserum to EF-Tu, except the samples analyzed in lanes A,B and I. Immunoprecipitation of samples G and H was done in the presence of 10 or 20 μg excess EF-Tu, respectively. Chemically tritiated EF-Tu [17,22] was used as standard (A,I). All samples were then subjected to SDS-PAGE followed by fluorography and autoradiography as described in section 2.

Fig.3 also shows the time course of methylation of the products precipitated with antiserum to EF-Tu. About 20% of the total methylated products were immunoprecipitated, indicating that several other components of the in vitro system are also modified.

To confirm that EF-Tu is indeed methylated under our experimental conditions, we used slab gel electrophoresis as shown in fig.4. In fig.4B, we can see the methylated products obtained with the ethionine system before immunoprecipitation. After precipitation of similar incubation reactions with antiserum to EF-Tu (C–E), a band of M_r ~43000 that comigrated with authentic EF-Tu (A,I) was found to be methylated. Other methylated products were also immunoprecipitated with the antiserum to EF-Tu. A similar result was obtained when using the methionine-limiting system (fig.4F,G,H). Addition of excess purified EF-Tu competes for the band of M_r 43000 in the antibody reaction (G,H). These results confirm the identity of the methylated protein with EF-Tu.

Some lower-M_r bands also immunoprecipitated with the antiserum to EF-Tu as occurred with the DNA-dependent system (fig.1). Some of these fragments were also competed out with pure EF-Tu although this is not readily apparent in fig.4. As mentioned before, the low-M_r bands may be generated by proteolysis during the in vitro incubations or more likely, during cell starvation for methionine, since incubations in the presence of phenylmethylsulfonyl fluoride did not alter the labeling pattern (not shown).

The significance of methylation of EF-Tu and most proteins has not yet been explained. It has been speculated that the modification of the factor

might be correlated with either the regulation of activity or of its synthesis, or it might fulfill a structural requirement of the molecule [5,8]. On the other hand, it has been recently proposed that the activity of the EF-1α from *M. racemosus* is regulated during germination through methylation of the protein [23].

The cell-free methylation systems developed in our laboratory will allow us to characterize for the first time the methyltransferase that modifies EF-Tu and to study in further detail the posttranslational modification of the elongation factor.

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