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Methylation of elongation factor EF-Tu affects the rate of trypsin degradation and tRNA-dependent GTP hydrolysis

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The role of methylation of elongation factor EF-Tu from *Escherichia coli* was investigated. The methylated factor was obtained from cells harvested at the late stationary growth phase. Submethylated forms of the factor were obtained from either bacteria grown to the mid-logarithmic phase or cells cultured in the presence of ethionine. When fully methylated EF-Tu was treated in the presence of trypsin, it showed a greatly reduced rate of degradation compared with both types of undermethylated EF-Tu factors. The methylation of EF-Tu appeared not to affect the rate of GDP and GTP exchange. However, aminoacyl-tRNA-stimulated GTPase activity determined in the presence of kirromycin was greatly increased when the EF-Tu was methylated.

Elongation factor; GTPase; Enzyme activity; Methylation

1. INTRODUCTION

During protein biosynthesis, the elongation factor EF-Tu recognizes, transports, and positions the codon-specified aminoacyl-tRNA to the A site of the ribosome [1–4] and plays an important role in the fidelity of the translation process [5]. EF-Tu has been described as being methylated in both *Escherichia coli* and *Salmonella typhimurium* [6–8], with either mono- or dimethyllysine at a unique site, Lys-56 [7]. More recently, we found that EF-Tu from chloroplasts was also methylated in *Euglena gracilis* (Toledo and Jerez, unpublished). The equivalent eukaryotic factor, EF-1a, is also methylated in several organisms [9–13]. The apparent conservation of this chemical modification suggests that it may have an important biological role.

In initial studies on methylation of EF-Tu from *E. coli*, we developed in vitro methylation systems in which different submethylated forms of the elongation factor were used as substrates [11]. Van Noort et al. subsequently suggested a role for methylation of EF-Tu [14], although the function of posttranslational methylation of the factor has not been documented in great detail. We describe here an analysis of some properties of methylated and undermethylated preparations of EF-Tu.

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2. MATERIALS AND METHODS

E. coli strains D-10 (*met, rel*), MRE 600 and LBE 1001 were kindly supplied by Drs D. Hayes and B. Kraal and were grown at 37°C in M-9 minimal medium supplemented with 20 μg methionine or 20 μg ethionine per ml. Cells grown in the presence of methionine were harvested at late stationary phase (*E. coli* LBE 1001) to obtain fully methylated EF-Tu (EF-TuL) and at mid-logarithmic phase (*E. coli* MRE 600) for submethylated EF-Tu (EF-TuM) according to Van Noort et al. [14]. On the other hand, *E. coli* D-10 grown in the presence of ethionine was used to obtain an alternative form of submethylated EF-Tu (EF-TuE) [15]. Cell-free extracts and purified EF-Tu preparations were obtained from each cell type as described [16,17].

The in vivo methylation of EF-Tu was determined essentially as described by Ferro-Luzzi and Niakido [6]. Briefly, *E. coli* D-10 cells were incubated for 8 min in the presence of 0.2 mg/ml chloramphenicol to inhibit protein biosynthesis. Thereafter, 2.2 μM [*methyl*-^{3}H]methionine (85 Ci/mmol) was added and incubation continued for an additional 30 min. The methylated products were analyzed by SDS-polyacrylamide gel

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electrophoresis (SDS-PAGE) essentially as in [15,18]. Methylated amino acids present in EF-Tu were assayed after immunoprecipitation of the factor as described [15].

EF-Tu-GDP was digested for the times specified with TPCK-treated trypsin at 0°C [enzyme/substrate ratio, 1:50 (w/w)] in 50 mM Tris-HCl buffer (pH 7.4) containing 5 mM $MgCl_2$, 1 mM EDTA, 0.1 mM DTT, 1 mM $CaCl_2$ and 10 μM GDP.

The dissociation rates for GDP and GTP and GTPase activities were determined essentially as in [14].

3. RESULTS AND DISCUSSION

3.1. *Methylation of EF-Tu during the growth phase of* E. coli

Previous reports have suggested that methylation of EF-Tu is correlated with the growth phase of bacteria [7,14]. To determine the methylating capacity of *E. coli* cells during their growth cycle, *E. coli* D-10 cells were harvested at various stages from a batch culture and subjected to treatment in the presence of chloramphenicol and [*methyl*-^{3}H]methionine as methyl group donor. Under these conditions, protein biosynthesis is completely inhibited, and therefore it is possible to detect incorporation of [^{3}H]methyl groups into the side chain of proteins [6]. The result of this analysis is depicted in fig.1. During logarithmic growth, EF-Tu was clearly able to undergo methylation, as shown by labeling of a 45 kDa protein band comigrating with EF-Tu. Methylation was maximal at about 4 h growth. This indicates that during the early stages of growth, the factor is a good substrate for the methyltransferases. Other low molecular mass products were also heavily methylated at 4–5 h growth. These probably correspond to ribosomal proteins such as L11 (17 kDa) and other ribosomal proteins which are also methylated [19–21]. When cells entered the stationary phase (8–10 h), no products were methylated during the 30 min incubations in the presence of [*methyl*-^{3}H]methionine and chloramphenicol. The most plausible explanation for this observation is that the methylation reaction itself was no longer taking place, since cells were still capable of incorporating the radioactive amino acid in the absence of chloramphenicol. This suggests that when cells had reached the stationary phase, they already contained fully methylated EF-Tu, as we have shown previously [15], since the EF-Tu present in this cell type was not methylated in vitro. These results, therefore, confirm the prediction of L'Italien and Laursen [7] of the dependence of methylation on growth phase.

Paper chromatographic analysis of the EF-Tu hydrolysate indicated the presence of both mono- and dimethyllysine, in agreement with previous findings [6,7,14,15] (not shown).

3.2. *Sensitivity of methylated and submethylated forms of EF-Tu towards tryptic degradation*

It has been suggested that a functional role of protein methylation might be to provide a protective effect against in vivo degradation by endopeptidases [22–24]. To ascertain the validity of this assumption in the case of EF-Tu, we have compared the rates of mild tryptic cleavage of methylated and submethylated EF-Tu-GDP preparations. In the first stage of mild tryptic cleavage of EF-Tu, the protein is cleaved at three positions: Arg-44, Lys-56 and Arg-58 [25], Lys-56 being the site of methylation. We observed that methylated EF-Tu was degraded more slowly than either of the submethylated EF-Tu samples (fig.2B,C,E,F). Thus, after 30 min proteolysis or earlier, both submethylated forms of EF-Tu are almost completely converted to the 39 kDa fragment, whereas the methylated factor requires much longer periods to undergo digestion to an equivalent extent. Since the rate of cleavage is dependent on the conformation of the protein, our results suggest that methylation of Lys-56 may alter the relative conformations of EF-Tu, conferring protection on the molecule against in vitro proteolysis. This finding is not in accordance with that of Van Noort et al. [14], since they did not observe methylation to exert an effect on the rate of EF-Tu degradation by trypsin. This discrepancy may be due to the different EF-Tu:trypsin ratio employed in each case, our value (1:50, w/w) being lower than theirs (1:30) [14]. In addition, L'Italien and Laursen [7] and Wittinghofer et al. [25] found two different peptides that contained Lys-56 when the factor was treated with trypsin. They explained the presence of these two peptides as being the consequence of differing degrees of methylation of the lysine.

3.3. *Role of methylation in EF-Tu activity*

No differences were observed in the rates of dissociation of GDP and GTP between submethylated and methylated forms of EF-Tu in

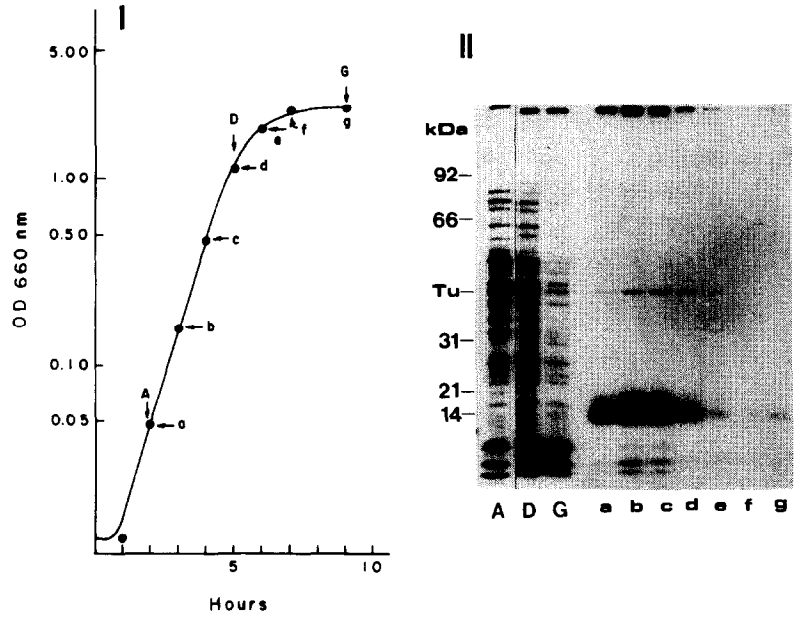

Fig.1. In vivo methylation of EF-Tu from *E. coli* during the growth cycle. (I) *E. coli* D-10 was grown in minimal medium supplemented with 20 μg/ml of methionine at 37°C. At the indicated times (arrows) fractions were taken and cells washed twice with the same medium but without methionine. (II) Washed cells from each fraction were suspended in medium without methionine and portions of each were incubated in either the presence (a–g) or absence (A,D,G) of 200 μg chloramphenicol/ml for 8 min at 30°C. Each aliquot containing the same amount of cells was then supplemented with [*methyl*-^{3}H]methionine (final concentration 2.2 μM) and incubation continued for 30 min at 30°C. Thereafter, cells were subjected to gel electrophoresis and radiolabeled proteins analyzed by 10% polyacrylamide SDS-PAGE followed by fluorography and autoradiography. (A,D,G) Total protein synthesized in the absence of chloramphenicol. (a–g) Incorporation of [^{3}H]methyl groups. Autoradiography involved exposure for 7 days at −70°C, except for lane A where the duration was 4 days.

either the absence or presence of EF-Ts (not shown). Similar findings were reported by Van Noort et al. [14]. However, significant differences were noted for the GTP-hydrolyzing activities of the modified factors in the presence of kirromycin and tRNA (fig.3). The GTPase activity of methylated EF-Tu was stimulated to a greater degree by aminoacyl-tRNA as compared to either of the submethylated EF-Tu preparations (EF-TuM, EF-TuE). Control experiments were carried out in which the GDP-dissociation rates for methylated and submethylated forms of EF-Tu were evaluated under the conditions employed for the GTPase assay. No difference in rates was observed, suggesting that the assay conditions per se did not contribute to variations in GTPase activities between the two EF-Tu forms (not shown).

Our results on GTPase activity contrast with those of Van Noort et al. [14], since they reported that EF-Tu isolated from *E. coli* in the stationary phase of growth had a reduced GTPase activity. Here, we employed two submethylated EF-Tu preparations (EF-TuM, EF-TuE), obtained via completely different procedures, and found that their GTPase activities were both less tRNA-stimulated as compared to the fully methylated form. In this regard, the methylated form of EF1-a from *Mucor racemosus* has also been shown to display greatly increased activity in protein synthesis vs the non-methylated protein [11].

Both EF-Tu [6–8,14,15] and ribosomes [19–21] are post-translationally modified, and it appears that these modifications may be directly related to the fact that *E. coli* moves from exponential growth to the stationary growth phase. Therefore, the observed in vitro effect of methylation upon EF-Tu GTP-hydrolyzing activity could be physiologically important, if it occurs in vivo, since GTPase activity is directly correlated with the fidelity of the ribosome-mediated translation process [5,26].

Although information concerning the possible

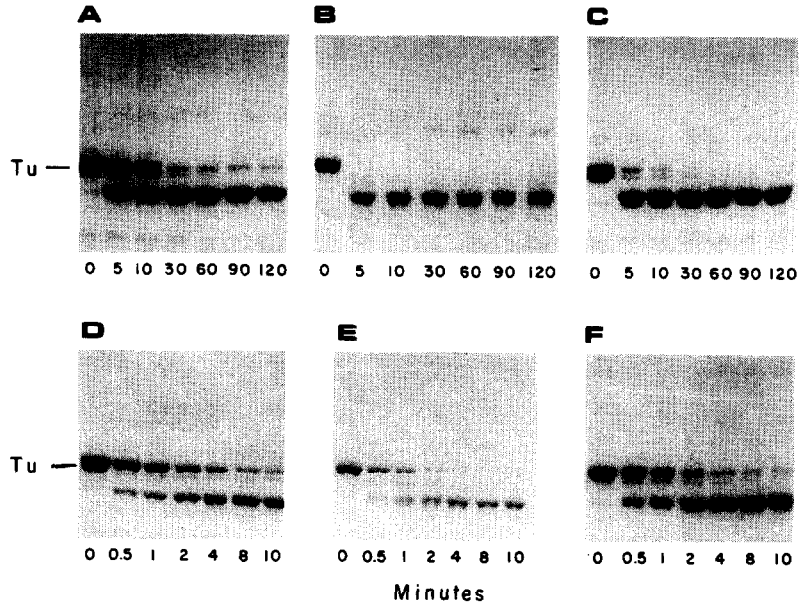

Fig.2. Tryptic degradation of methylated and submethylated EF-Tu forms. 50 μg methylated EF-TuL (A,D), submethylated EF-TuM (B,E) and submethylated EF-TuE (C,F) were treated with trypsin at 0°C. At the times indicated, aliquots containing 3 μg of each kind of EF-Tu were mixed with an equal volume of electrophoresis sample buffer and then boiled for 5 min. All samples were then subjected to 10% polyacrylamide SDS-PAGE. The gel was stained with Coomassie blue. Only the regions of interest are shown.

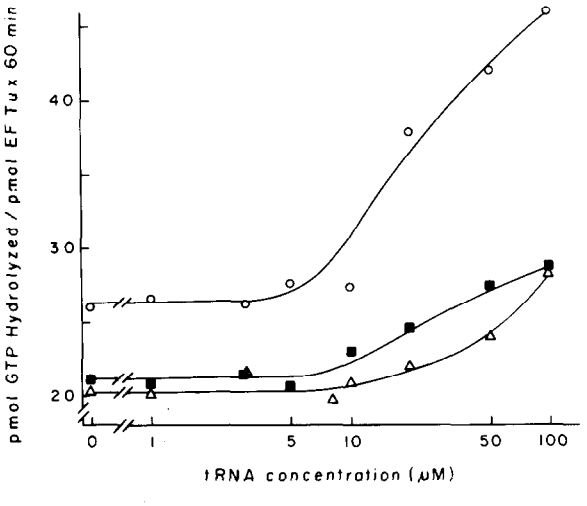

Fig.3. Effect of methylation of EF-Tu on GTPase activity of the factor. The effect of different aminoacyl-tRNA concentrations was tested on the GTPase activity of 100 pmol EF-TuL (○—○), EF-TuM (△—△), and EF-TuE (■—■) in the presence of 50 μM kirromycin and 6 nmol [^{3}H]GTP (10 Ci/mmol). The hydrolyzed GTP was determined by assaying [^{3}H]GDP isolated by polyethylenimine thin-layer chromatography.

effect of chemical modification of EF-Tu is sparse at present, our results suggest that the methylation of EF-Tu may play a protective role against in vivo degradation and perhaps may carry out a fine modulation function during the recognition of the aminoacyl-tRNA in protein synthesis.

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