LOCATION OF THE SITE OF METHYLATION IN ELONGATION FACTOR Tu

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Received 21 August 1979
Revised version received 2 October 1979

1. Introduction

Elongation factor Tu (EF-Tu) has a variety of functions and properties that has made it the subject of numerous studies in recent years. Besides playing a key role in protein biosynthesis [1], EF-Tu is a regulator of RNA polymerase activity [2], is a component of Qβ RNA polymerase [3], and is coded for by two genes [4]. EF-Tu also appears to be associated with bacterial membranes [5]. The amino acid sequence of EF-Tu has been completed [6] and several X-ray crystallographic studies are underway [7–9]. EF-Tu in *Escherichia coli* and *Salmonella typhimurium* has been reported methylated in vivo to form mono- and dimethyllysine [10]. Here we show that EF-Tu from *E. coli* contains a single lysine residue which is methylated and that it is probably located in an exposed position on the surface of the protein.

2. Materials and methods

[^{3}H]Methylated EF-Tu was supplied by Dr G. Ferro-Luzzi Ames as Coomassie blue-stained bands on a dried SDS–polyacrylamide slab gel [10], and unlabeled EF-Tu, purified as in [11], was provided by Dr D. Miller. Both samples were from *E. coli* B originating in Dr Miller's laboratory.

Bands of [^{3}H]methylated EF-Tu were cut from the dried gels and were allowed to swell in 200 μl 0.1 M NH_4HCO_3 (pH 7.8) at room temperature for 1 h. Unlabeled carrier EF-Tu (500 μg), denatured with 6 M guanidinium chloride and extensively dialyzed against water, was then added to the gel slices. The protein was digested by adding 10 μg trypsin and incubating the mixture at 37°C. After 4 h the reaction mixture was centrifuged and the supernatant was removed. The precipitate was then resuspended in 300 μl of 0.1 M NH_4HCO_3 and redigested with 5 μg trypsin for 16 h. The supernatants from both digestions were combined, digested again with 5 μg trypsin and lyophilized. The peptide mixture containing [^{3}H]methylated peptides (~45 000 dpm/map) was subjected to two-dimensional peptide mapping on cellulose thin-layer chromatographic (TLC) plates [12]. The peptide maps were then sprayed with 0.001% fluorescamine in acetone after adjusting the pH of the plate by dipping in a 5% pyridine in acetone solution [13]. The radioactive peptides were located by exposure of the TLC plate to LKB Ultrofilm for 24 h. The radioactive spots were then scraped from the TLC plate, and the peptides were extracted with 6 M HCl and hydrolyzed at 110°C for 20 h. The peptides could then be identified by their amino acid compositions.

The site of methylation in non-radiolabeled EF-Tu was determined by analysis of cyanogen bromide and tryptic peptides (the isolation of which will be described elsewhere) for mono- and dimethyllysine. These amino acids could readily be detected by elution of the 6 mm column of the amino acid analyzer (Beckman 119CL) with 0.35 M sodium citrate (pH 5.5) at 30°C. A similar system has been described [14]. N^{ϵ}-Methyllysine and N^{ϵ}-N^{ϵ}-dimethyllysine used as standards were purchased from Sigma and Vega, respectively.

3. Results

Autoradiography of the [^{3}H]methylated EF-Tu

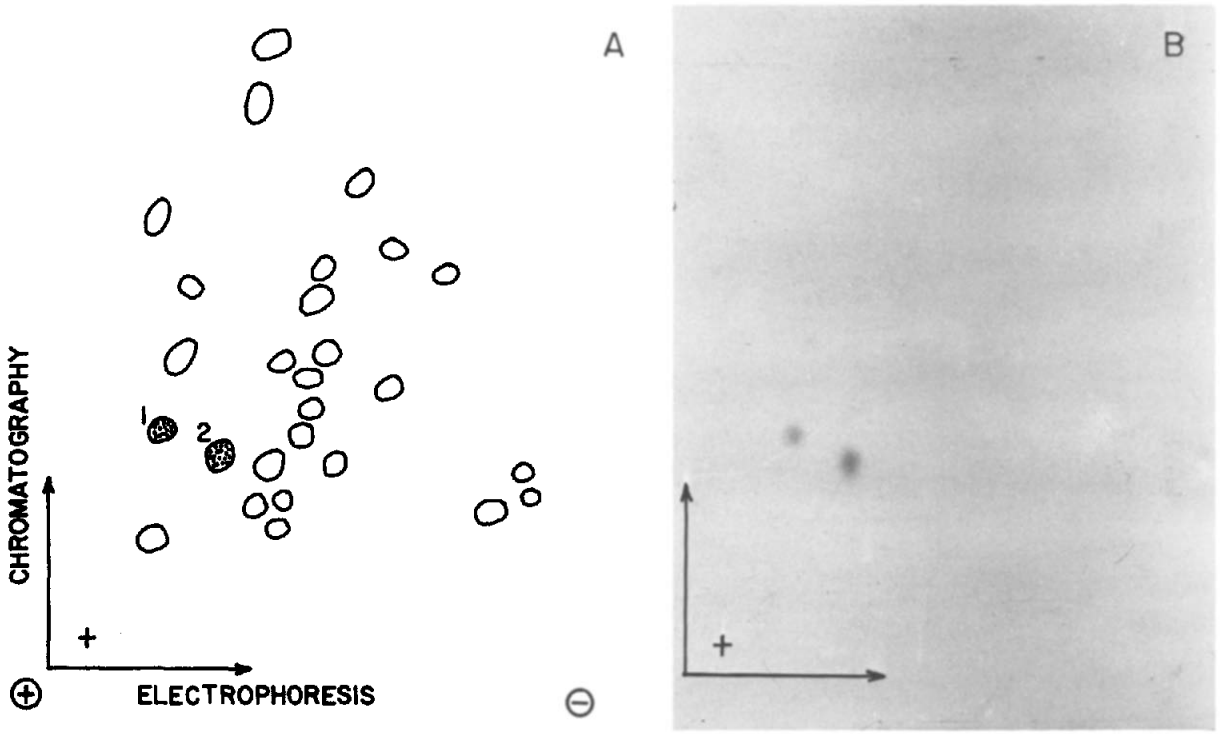

Fig.1. Peptide map of trypsin digested [^{3}H]methylated EF-Tu on a cellulose (EM Lab./no. 5502) TLC plate. First dimension, chromatography in pyridine/1-butanol/acetic acid/water (50:75:15:60, by vol); second dimension, electrophoresis in pyridine/acetic acid/acetone/water (20:40:150:730, by vol.) at 400 V for 3 h. (A) Schematic representation of the map after staining with fluorescamine; stipled spots indicate location of radioactivity; spot 1 has the sequence AFDQIDNAPEEK(CH_3) and spot 2, AFDQIDNAPEEK(CH_3)$_2$AR. (B) Autoradiogram of the same map.

tryptic map revealed only two radioactive peptides (fig.1). In addition, liquid scintillation counting of all fluorescamine-positive spots, as well as non-staining portions of the plate, showed that $\geqslant 95\%$ of the radioactivity could be accounted for by the same two spots. Amino acid analysis of the spots gave compositions that corresponded to the overlapping sequences:
Ala–Phe–Asp–Gln–Ile–Asp–Asn–Ala–Pro–Glu–Glu–Lys

and

Ala–Phe–Asp–Gln–Ile–Asp–Asn–Ala–Pro–Glu–Glu–Lys–Ala–Arg

for spots 1 and 2, in fig.1, respectively, reported [15]. Analysis for methyllysines showed that spot 1 contained monomethyllysine and spot 2, mostly dimethyllysine. Thus the site of methylation appears to be a single lysine, specifically Lys_{56} in the EF-Tu sequence [6].

We also were able to identify the position of methylation using nonradioactively labeled protein. Amino acid analysis of intact EF-Tu showed both mono- and dimethyllysines in a ratio of ~1.5 : 1. The only cyanogen bromide peptide that contained methyllysines was CB-1 [16], which comprises residues 1–91. Analysis of tryptic peptides of CB-1 isolated by ion-exchange chromatography showed three peptides which contained methyllysines (see table 1).

Peptide CB1-T1 corresponds to spot 1 (fig.1) and contains C-terminal lysine and monomethyllysine, whereas CB1-T3 (spot 2), which contains mostly dimethyllysine, has the C-terminal sequence

Table 1
Methyllysine content of peptides[a]

Peptide	% in peptide			Relative amount of peptide Isolated	Relative amount isolated[c]				MML
	Lysine	MML[b]	DML[b]		Lysine	MML	DML	dpm	DML
EF-Tu	n.d.	60	40	–	–	–	–	–	1.5
CB-1	n.d.	85	15	–	–	–	–	–	5.7
CB1-T1[d]	55	45	0	10	5.5	4.5	0	–	
CB1-T2	80	20	0	0.5	0.4	0.1	0	–	
CB1-T3	24	4	72	1	0.24	0.04	0.72	–	
Sum of CB1-T peptides					6.1	4.6	0.7		6.6
spot 1[e]								4699	7.0[f]
spot 2[e]								1344	

[a] Assuming the ninhydrin color yields for the methyllysines to be the same as for lysine, which is only approximately true
[b] MML, N^{ϵ}-monomethyllysine; DML, $N^{\epsilon},N^{\epsilon}$-dimethyllysine
[c] Relative amount of peptide isolated times % in peptide
[d] The sequence for CB1-T1 is AFDQIDNAPEEK; for CB1-T2 and CB1-T3, AFDQIDNAPEEKAR
[e] cf. fig.1; less radioactive material was applied to the map prepared for scintillation counting
[f] Ratio calculated assuming spot 1 contains MML and spot 2 only DML and that the specific activity for DML is twice that of MML

$Lys(CH_3)_2$–Ala–Arg. The occurrence of two peptides is consistent with the known specificity of trypsin, which cleaves at monomethyllysine, but not at dimethyllysine [17]. The presence of lysine in CB1-T3 and the occurrence in low yield of CB1-T2, indicate that cleavage even of the unmodified Lys-Ala bond by trypsin was incomplete.

The numbers listed in table 1 can be regarded only as approximate, because of incomplete recovery of peptides and deficiencies in the analytical methods, but they do show some internal consistencies that indicate that we are not dealing with an artifact. The ratios of mono- to dimethyllysine in CB-1 and in the sum of the tryptic peptides from CB-1 are similar, 5.7 and 6.6, respectively. These values are close to the ratio of 7.0 found by counting the radioactive spots 1 and 2. On the other hand, a ratio of 1.5 was found for EF-Tu itself. The differences probably reflect the fact that all of the EF-Tu samples had different histories; for example the radioactive EF-Tu was isolated from *E. coli* grown in the presence of chloramphenicol [10] and the non-labeled material was not. Only the CB-1 peptides were from the same batch. The similarity in ratios between the CB-1 peptides and spots 1 and 2 is probably coincidental. It seems quite likely that the degree of methylation is dependent on growth conditions and the time at which cells are harvested. Another indication that this may be true is that 80% of EF-Tu was reported methylated [10]. As can be calculated from the data for the CB-1 tryptic peptides (table 1), our results indicate that ~45% of Lys_{56} residues are methylated.

4. Discussion

The results presented here confirm the report [10] that EF-Tu is methylated in vivo at lysine, and show further that the only site of methylation is Lys_{56} in the EF-Tu sequence. It is possible that there are secondary sites, but the degree of methylation must be very low, probably $< 5\%$. However, our findings and those in [10], contradict those in [18], where no methylation of any non-ribosomal proteins in *E. coli* was detected. This discrepancy remains unexplained.

In [15] we noted that the bond (Arg_{58}–Gly_{59}) in EF-Tu that is rapidly cleaved by trypsin [19,20] is probably located on an exposed loop or turn on the surface of the EF-Tu molecules. The finding that the methylation site, which must be accessible to a methylase, is only two residues away from the cleavage site supports this hypothesis.

Yet unexplained is the significance of methylation

in EF-Tu, or, for that matter, most other proteins. EF-Tu is the only methylated intracellular, non-ribosomal protein found in prokaryotes so far. On the other hand, many ribosomal proteins are methylated [21,22], several of which (L3, L7/L12, L11, L16 and L33) appear to be near either the peptidyl transferase center [23–25] or the EF-Tu · tRNA binding site [23,26] of ribosomes. Although the site of tRNA binding in EF-Tu is not yet known, there is evidence that loss of the 14-residue fragment containing the methyllysine (residues 45–58; CB1-T3) by limited trypsinolysis results in loss of aminoacyl-tRNA binding activity (F. Jurnak, personal communication). Perhaps methylation acts as a mechanism for 'fine tuning' of RNA binding sites: the protein functions without methylation, but a little better with it. At present, however, the role of methylation remains a tantalizing mystery.

Acknowledgement

This work was supported by National Science Foundation grant no. PCM 76-04361.

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