A KIRROMYCIN RESISTANT ELONGATION FACTOR EF-Tu FROM *ESCHERICHIA COLI* CONTAINS A THREONINE INSTEAD OF AN ALANINE RESIDUE IN POSITION 375

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1. Introduction

The peptide chain elongation factor EF-Tu from *Escherichia coli* is an allosteric protein with multiple activities in the bacterial cell. Besides its function in protein synthesis [1,2], it acts as a subunit of the enzyme Qβ RNA replicase [3]. Its extremely high intracellular abundance [4,5] and its peripheral association with the inner membrane of the *E. coli* cell [4] have led to speculations about an additional structural role for this remarkable protein [6,7].

Two unlinked genes coding for EF-Tu have been identified on the *E. coli* chromosome [8–10]. One designated *tufA* maps at 72 min, the other, designated *tufB*, at 88 min. Their nucleotide sequences have recently been elucidated [11,12]. The primary structures of the corresponding gene products designated EF-TuA and EF-TuB, respectively, were thus found to be identical, except for the C-terminal amino acid residue (Gly or Ser). This is in accordance with the directly determined primary structure of the mixture of the two proteins [13,14].

EF-Tu interacts with a great number of ligands and in some cases this interaction has been correlated with conformational changes of the polypeptide chain [2,15,16]. To study the relation between the structure and function of this multifunctional and allosteric protein, we have isolated a number of *E. coli* mutants with an altered EF-Tu. We took advantage of the fact that the antibiotic kirromycin binds to the factor in an 1:1 molar ratio [17]. This binding to EF-Tu · GDP induces an EF-Tu · GTP-like conformation which prevents the release of the factor from the ribosome, thus blocking protein synthesis [15,18,19].

Expression of a kirromycin-resistant phenotype requires the alteration of both *tufA* and *tufB* [10,20, 21]. Inactivation of *tufB* by insertion of bacteriophage Mu or by an amber mutation enabled us to isolate single gene products derived from either wild-type *tufA* (designated EF-TuA$_S$) or kirromycin resistant *tufA* (designated EF-TuA$_R$). Both protein species have the same relative molecular mass (M_r) and isoelectric point [22]. EF-TuA$_R$ has a strongly reduced affinity for kirromycin, but its function in polypeptide synthesis appeared to be normal [17,22,23]. On the other hand, if EF-TuA$_R$ replaces its wild-type counterpart in Qβ replicase the enzyme is rapidly inactivated and displays an apparent increase in template specificity [24].

Here we show that Ala-375 of EF-TuA$_S$ is substituted by Thr in EF-TuA$_R$. The importance of this substitution for structure and function of the enzyme is discussed.

2. Materials and methods

2.1. *Materials*

EF-TuA$_S$B$_S$ (wild-type EF-Tu, a mixture of *tufA* and *tufB* gene products) was isolated from *E. coli* B, and EF-TuA$_R$ (kirromycin-resistant *tufA* gene product) from the *E. coli* mutant strain LBE 2045 [10] as in [25]. All other materials were the same as used in [26].

2.2. *Methods*

EF-Tu was digested with trypsin at an enzyme/substrate ratio of 1/100 (w/w) in 0.1 M NH_4HCO_3, pH 7.8 at 37°C for 4 h. After lyophilization, fingerprint analysis of the mixture of peptides was performed as in [26]. Amino acid analysis of the eluted peptides and their sequence determination by means of the dansyl-Edman degradation procedure were done as in [27].

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3. Results

3.1. *Amino acid substitution at position 375*

Fingerprint analysis of the tryptic peptides obtained from a wild-type mixture of EF-TuA_S and EF-TuB_S and from mutant EF-TuA_R are presented in fig.1. Electrophoretic runs were performed at pH 3.5 (upper part) and at pH 6.5 (lower part). After fluorescamine staining all the visible peptides were eluted for amino acid analysis. This enabled their assignment in the peptide chain, according to the primary structure in [14]. About 70% of all the amino acids of the EF-Tu mole-

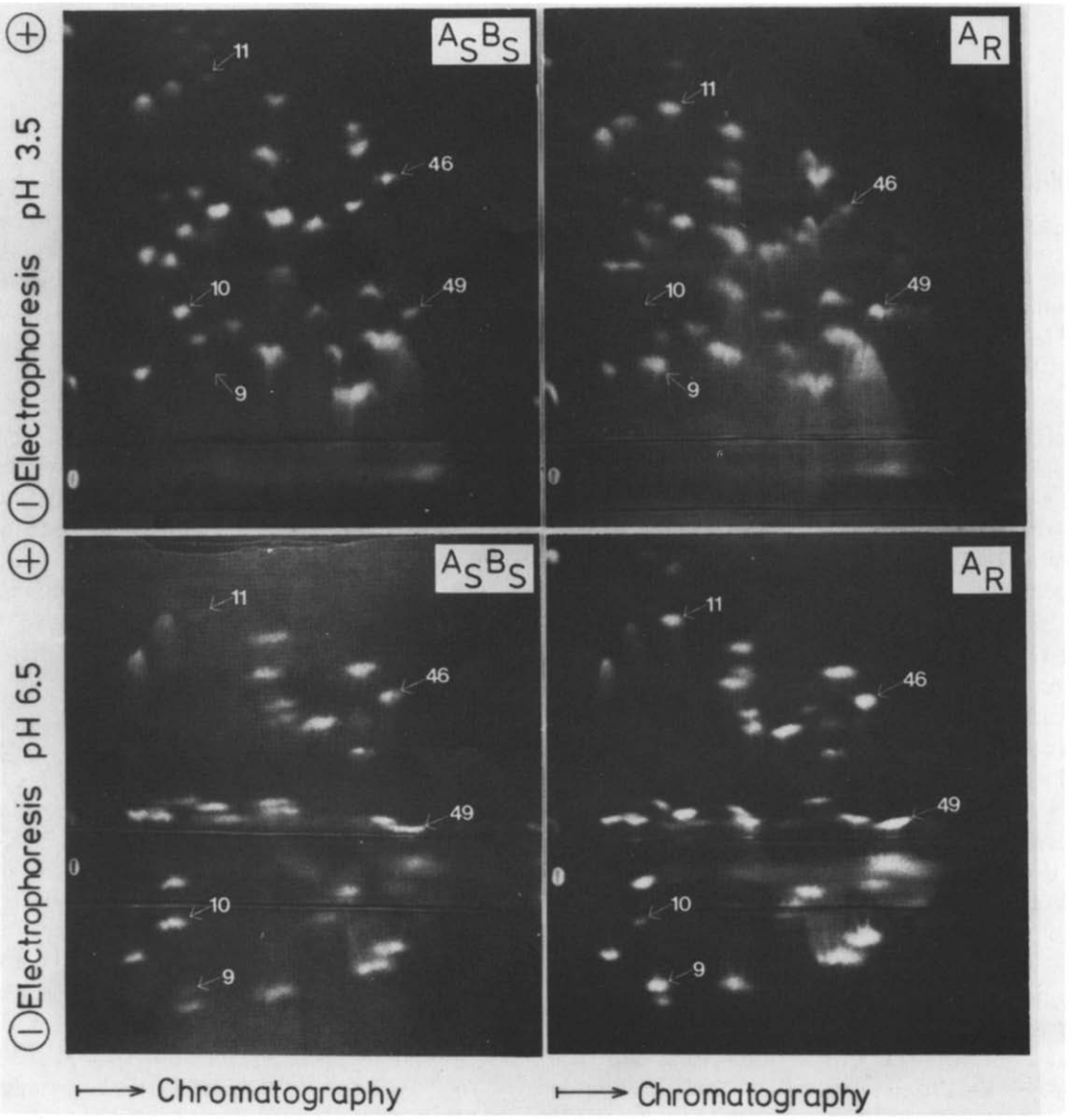

Fig.1. Fingerprint analysis of the tryptic peptides of wild-type EF-Tu(A_SB_S) and kirromycin resistant EF-Tu(A_R). About 2 mg protein was digested with trypsin and spotted on Whatman 3 MM paper. After descending chromatography with butanol/pyridine/acetic acid/water (15/10/3/12, by vol.) the upper two papers were subjected to electrophoresis at pH 3.5 (pyridine/acetic acid/water, 1/10/90, by vol.) and the lower two to electrophoresis at pH 6.5 (pyridine/acetic acid/water, 25/1/225, by vol.). On the left side of the origin, 20 nmol Phe and Lys were spotted as markers during the electrophoresis.The peptides were visualized with fluorescamine. The numbers indicated in the figure refer to the numbering of tryptic peptides in [14]; T9, positions 45–56; T10, 45–58; T11, 57–58; T46, 374–378; T49, 390–393. (–) = cathode; (+) = anode.

Table 1
Amino acid compositions of the tryptic peptide T46 (positions 374–377) from EF-TuA_SB_S and EF-TuA_R

Amino acid[b]	EF-TuA_SB_S		EF-TuA_R	
	I[a]	II[a]	I[a]	II[a]
Thr			1.1	0.9
Ala	0.9	1.1		
Ile	1.0	0.8	0.9	0.9
Phe[c]	0.4	0.5	0.4	0.4
Arg	1.1	1.1	1.0	1.2
Peptide yield (%)	20	15	20	24

[a] I and II indicate isolation from a fingerprint with electrophoresis in the second dimension at pH 3.5 and 6.5, respectively (see fig.1)
[b] Results are expressed as residues/molecule; data <0.1 are not given
[c] The reduced recovery of the NH_2-terminal amino acid is due to the fluorescamine staining (see fig.1)

cule could thus be accounted for. Comparison of the amino acid compositions of the EF-TuA_R peptides with those of wild-type peptides revealed that the alanine residue in T46 from EF-TuA_SB_S is replaced by threonine in T46 from EF-TuA_R (table 1). Such a replacement agrees in the finding [22] that EF-TuA_SB_S and EF-TuA_R do not differ in isoelectric point and with the equal position of T46 in the corresponding fingerprints (fig.1). Dansyl-Edman degradation of mutant T46 yielded the sequence Phe–Thr–Ile–Arg. It is concluded that position 375 in EF-TuA_R is occupied by threonine.

3.2. *Methylation of lysine residue 56*

Several authors [13,14,28,29] have reported that Lys-56 could be methylated and that the extent of modification varied among the preparations. Fig.1 shows that peptides T9 (residues 45–56) and T11 (residues 57–58), which are clearly visible in the fingerprints of EF-TuA_R, have almost vanished entirely from the prints of the wild-type factor. On the other hand peptide T10 (residues 45–58) is conspicuous in the latter fingerprint, but barely visible in that of EF-TuA_R. Amino acid analysis of wild-type T10 showed the presence of mainly ε-*N*,*N*-dimethyl-lysine and the virtual absence of lysine. Trypsin only hydrolyzes the peptide bond between the residues 56 and 57 when Lys-56 is not methylated. In EF-TuA_R peptide T10 is almost completely fragmented, giving rise to T9 and T11 (fig.1). In this case only 15% of Lys-56 appeared to be methylated to ε-*N*-methyl-lysine. ε-*N*,*N*-Dimethyl-lysine was completely absent in EF-TuA_R.

In another preparation of EF-TuA_SB_S (isolated from the Q13 strain of *E. coli*) we exclusively found non-methylated lysine at position 56 (not shown). The two preparations of EF-TuA_SB_S with 0% and 100% ε-*N*,*N*-dimethyl-lysine were found to be equally active in sustaining polypeptide synthesis in vitro (not shown).

3.3. *The COOH-terminal amino acid residue*

Amino acid analysis of the COOH-terminal tryptic peptide T49 from EF-TuA_R revealed the presence of valine, leucine and glycine. No serine was detected. This is in agreement with the fact that EF-TuA_R is a single-gene product derived from *tufA* [10,11,22].

4. Discussion

The codon corresponding with Ala-375 in both *tufA* and *tufB* is GCA [11,12]. Apparently the substitution of Ala-375 by Thr (ACA) is caused by a transition (G–C → A–T), in accordance with the fact that ethyl methane sulphonate was used originally for mutagenesis [10,30,31]. Probably the mutation of EF-TuA_R is restricted to this transition although we have not analyzed the tryptic peptides T7, T14, T16, T18, T23 and T24 (cf. [14]), which could not be identified on the peptide map of fig.1. Additional mutations, however, seem unlikely considering the frequency (10^{-8}) of spontaneous mutations of the *tufA* gene to kirromycin resistance in cells which harbour already a mutated *tufB* gene [10]. Similarly, streptomycin resistance in *E. coli* appeared by single amino acid substitutions [32].

X-Ray diffraction patterns at 4 Å resolution of EF-TuA_R · GDP crystals did not reveal differences of the unit cell and the symmetry as compared to the corresponding parameters of wild-type EF-Tu [33]. This means that the 2 molecules are very similar or if differences exist, they are very subtle.

X-Ray diffraction studies of wild-type EF-Tu · GDP crystals in [34–36] have led to a tentative model in which two domains can be distinguished: a rather tight domain with considerable α-helical characteristics; and a less well defined domain separated from the tight domain by a cleft. The tight domain com-

prises approximately residues 61–240 [38] and contains the nucleotide binding site [34,37,38] situated on its surface and opposite to the rest of the molecule. Therefore a direct interaction between nucleotide and Ala-375 seems unlikely.

The following data suggest that Ala-375 is located in a region, which is profoundly involved in the allosteric control of the EF-Tu molecule. Various authors have provided evidence that the NH_2-terminal region contains a binding site for the acceptor stem of aminoacyl-tRNA [35,39]. Upon binding of kirromycin to the EF-Tu · GDP complex conformational changes can be monitored in this part of the molecule by measuring an increased accessibility for tryptic cleavage at Arg-44, Lys-56 and Arg-58 [15]. Furthermore, the EF-Tu · GDP · kirromycin complex displays an enhanced affinity for aminoacyl-tRNA [18,23]. Conformational alterations also become apparent in the nucleotide binding site on the tight domain when the antibiotic binds to EF-Tu: the binding affinities of GTP and GDP change [15,22,40] and GTP hydrolysis is induced [16,41,42]. On the other hand, replacement of GDP by GTP opens the aminoacyl-tRNA binding site [1,2] and alters the conformation around residues 44, 56 and 58 in a way very similar to that observed after kirromycin binding [15]. Presumably, movement of the 2 major domains relative to each other alters the orientation of the NH_2-terminal region significantly. If so, this would implicate that the cleft between the two domains is involved in the alllosteric effect by kirromycin. Substitution of Ala-375 by Thr reduces the affinity of EF-Tu for the antibiotic by ~2 orders of magnitude [17,22,23]. Steric hindrance could be responsible for this reduction in affinity. Another possibility is that the amino acid replacement primarily affects the allosteric changes accompanying for kirromycin binding. It is tempting to speculate that the allosteric site in which Ala-375 may be located in fact is in the cleft between the two domains of EF-Tu, but crystallographic data have to be awaited before such a suggestion can be substantiated.

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