ALLOSTERIC CHANGES OF THE GUANINE NUCLEOTIDE SITE OF ELONGATION FACTOR EF-Tu

A comparative study of two kirromycin-resistant EF-Tu species

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1. Introduction

The prokaryotic elongation factor EF-Tu is a multifunctional protein, which is able to interact with a considerable number of biological macromolecules. During the elongation cycle of protein biosynthesis it plays an essential role in the correct placement of aminoacyl-tRNA onto the ribosome–messenger RNA complex [1,2]. In *Escherichia coli* two genes, *tufA* and *tufB*, code for EF-Tu [3–5]. The primary structure of the polypeptide chain of these two gene products is identical, only the C-terminal residue is different [6]. The evolutionary pressure on the EF-Tu molecule appears to be extremely high; even the DNA base sequence of *tufA* shows homology over a wide variety of species; outside the gene this homology is lost immediately [7].

We have isolated mutant *E. coli* strains resistant to the antibiotic kirromycin [5,8]. This antibiotic inhibits the elongation cycle of protein biosynthesis by preventing the release of EF-Tu from the ribosome after hydrolysis of GTP, thus blocking the ribosome on the messenger RNA [9–11]. Kirromycin strongly increases the binding affinity of EF-Tu for GTP [12] and gives rise to a turnover GTPase activity of the protein [9].

The EF-Tu–guanine nucleotide interaction of various wild-type EF-Tu's and mutant EF-Tu_{D2216} in the presence of several ligands such as kirromycin, the elongation factor EF-Ts, Phe-$tRNA^{Phe}$ and/or ribosomes, and under the influence of mono- and divalent cations has been studied [12–14,17]: the GTPase centre of the molecule is activated in the presence of increasing monovalent cation concentrations; this activation is stimulated by NH_4^+ up to a concentration of 800 mM, where it reaches a plateau [14]. The most important difference found with regard to the mutant EF-Tu_{D2216} was an intrinsically increased affinity for GTP. This mutant protein also displays considerable GTPase activity in the absence of any macromolecular effector, whereas the wild-type does so only in the presence of the antibiotic [12]. It was later demonstrated that the endogenous GTPase activity of the mutant was sustained by the increased affinity for GTP [14].

The two EF-Tu species resistant to kirromycin (LBE2045 and D2216) have one mutation site in common: amino acid residue 375 of the polypeptide chain [15,16]. While the wild-type contains an alanine in that position [6], EF-Tu_{D2216} contains a valine [16] and EF-$Tu_{LBE2045}$ a threonine [15].

Here, we have made a comparative study of EF-Tu from wild-type *E. coli* strains and from the mutant strains LBE2045 and D2216 (table 1). First we compared their sensitivities to the antibiotic kirromycin and their kinetic constants for EF-Tu–guanine nucleotide complex formation and dissociation.

To get a deeper insight into the consequences of the mutation for the conformation of the protein we followed the behaviour of the different EF-Tus as a function of the NH_4^+ concentration in the two reactions most characteristically changed:

Abbreviation: EF-Tu, elongation factor EF-Tu; subscripts refer to the strain from which the protein was isolated

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The dissociation of the EF-Tu–GTP complex:

$$\text{EF-Tu–*GTP} + \text{GTP} \rightarrow \text{EF-Tu–GTP} + \text{*GTP} \quad (1)$$

The activation of the GTPase centre as measured by the GTPase activity of the pre-formed EF-Tu–GTP complex in the absence of any macromolecular effector or kirromycin:

$$\text{EF-Tu–GTP} \rightarrow \text{EF-Tu–GDP} + P_i \quad (2)$$

For each of the two mutants we observed characteristic differences in both these reactions, emphasizing the pivotal role of the amino acid residue at position 375 in the regulation of the EF-Tu activities.

2. Materials and methods

EF-Tu from strains MRE600 and LBE2045 was isolated as in [18]; EF-Tu from strains B, D22 and D2216 was isolated as in [10]. Nucleotide-free EF-Tu was prepared as in [12]. The specific activities of [γ-^{32}P]GTP and [^{3}H]GDP were determined by isotopic dilution.

Kirromycin resistance was tested by poly(U)-directed poly(Phe) synthesis in a system in which EF-Tu was present in rate-limiting amounts. Dissociation rate constants, association rate constants and GTPase activity were determined as in [12]. Details are in the legends to tables and figures.

Before measuring the activation of the GTPase centre, the EF-Tu–GTP complex was formed from nucleotide-free EF-Tu and a 4–8 times excess of GTP to saturate the enzyme with its substrate. In this system hydrolysis of GTP is the rate-limiting step [21]. First order plots of hydrolysis of the complex, reaction (2), showed a clear linear relationship during the initial 10–15 min, also for EF-Tu$_{D2216}$. Disturbance of the one round reaction by turnover GTPase activity, because of the much higher affinity of EF-Tu$_{D2216}$ for GTP, is apparent only after longer times. Dissociation rate constants were all determined at 5°C; at this temperature the velocity can be determined more precisely because of the slower dissociation and it does not need correction for interfering intrinsic GTPase activity. This process proceeds at a faster rate at higher temperatures, but the equilibrium constant is essentially not affected [12], so rate constants of the dissociation reaction at 5°C and the GTPase activity at 37°C can be compared directly.

Table 1
Survey of *E. coli* strains used in the present research project, with their characteristic kirromycin resistance values in poly(U)-directed poly(Phe) synthesis

E. coli strain	Kirromycin phenotype	EF-Tu species	Kirromycin concentration inducing 50% inhibition of poly(Phe) synthesis
MRE600	Sensitive	$A_S B_S$	0.07 μM
B	Sensitive	$A_S B_S$	n.d
D22	Sensitive	$A_S B_S$	0.1 μM
D2216	Resistant	A_R[a]	20 μM
LBE2045	Resitant	A_R[b]	6 μM

[a] Originally the EF-Tu produced by D2216 was designated to be a *tufB* product by genetic evidence [8]. In [16] however, it appeared to be a *tufA* product from end-group determination

[b] *tufB* of LBE2045 was inactivated by insertion of bacteriophage Mu [5,20]

'A' and 'B' refer to *tufA* and *tufB*, respectively, the genes encoding EF-Tu; subscripts, S, sensitive; R resistant

Each assay (75 μl) contained 50 mM imidazole acetate (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 0.4 mM GTP, 0.3 mM ATP, 2.6 mM phosphoenylpyruvate, 1 μg pyruvate kinase, 4 μg poly(U), 40 pmol ribosomes, 120 pmol [^{14}C]Phe (900 cpm/pmol), 7 pmol elongation factor G, 88 μg $tRNA_{total}$, 10 pmol elongation factor EF-Ts, 10 pmol EF-Tu and $tRNA^{Phe}$-synthetase. [Kirromycin] varied from 0–0.5 mM. The incubation time was 10 min at 30°C

3. Results

The kirromycin resistance values for the various EF-Tu species as measured in the poly(U)-directed poly(Phe) synthesis are given in table 1. The difference found between the wild-type EF-Tus is probably not significant. EF-$Tu_{LBE2045}$ is ~70-times more resistant than wild-type, EF-Tu_{D2216} ~230-times. The difference between the two mutants is very reproducible under the conditions used.

Table 2 presents our data concerning the apparent association (k'_{+1}) and dissociation (k'_{-1}) constants of the interaction between EF-Tu and GTP and GDP; from these two rate constants the apparent equilibrium constant ($K'_d = k'_{-1}/k'_{+1}$) was calculated. The values of the rate constants for wild-type EF-Tu_B are in good agreement with those reported for wild-type EF-Tu_{D22} [13], as was checked at 0°C and 60 mM NH_4Cl (not shown); these values are ~50% higher than those in [12,14]. With dissociation of EF-Tu_{D2216}–GTP we observed a faster initial phase, which was not dependent on $[NH_4^+]$ and disappeared at higher ionic strengths; the majority of the complex dissociated at this rate. The interaction of EF-$Tu_{LBE2045}$ with GDP did not differ significantly from that of the wild-type and of D2216, determined in [12,13]. However, EF-Tu_{D2216} displays a much higher affinity for GTP than wild-type EF-Tu. This is caused by a retarded dissociation of the nucleotide from the complex.

However, the affinity of EF-$Tu_{LBE2045}$ for GTP is very close to that of wild-type EF-Tu.

It thus became of interest to know how the mutations affect the GTPase centres of the proteins. Fig.1 and table 3A show the GTPase activities as a function of increasing $[NH_4^+]$. At all concentrations EF-Tu_{D2216} appears to be affected most. EF-$Tu_{LBE2045}$ did not show any difference from wild-type EF-Tu in the absence of added NH_4^+. Upon raising the $[NH_4^+]$, however, the activation of the GTPase centre by the mutation becomes increasingly apparent. This activation does not reach the level observed with EF-Tu_{D2216}. The pattern of activation by NH_4^+ in the latter case is similar to wild-type in contrast to that seen with EF-$Tu_{LBE2045}$ (compare the two last columns of table 3A).

This raised the question whether the affinity of EF-Tu for GTP would exhibit a similar pattern. Table 3B shows that this is not the case: the ratio of the dissociation rate constants of both mutant EF-Tu species *vs* wild-type remains the same when the $[NH_4^+]$ is increased (compare the last two columns of table 3B).

4. Discussion

These data and those in [15,16] show that amino acid replacement at position 375 of the EF-Tu polypeptide chain not only affects the interaction with the antibiotic kirromycin but also that with GTP.

Table 2
Apparent association and dissociation rate constants for the complex between EF-Tu from various strains and the guanine nucleotides GTP and GDP at 50 mM of NH_4^+ and 5°C

Nucleotide	EF-Tu species	Apparent association rate constant $10^{-4}.k'_{+1}$, ($M^{-1}.s^{-1}$)	Apparent dissociation rate constant $10^{4}.k'_{-1}$, (s^{-1})	Apparent equilibrium constant $K'_d = k'_{-1}/k'_{+1}$, (nM)
GTP	Wild-type, B	2	154	770
	LBE2045	2	115	575
	D2216	2	18.2	91
GDP	Wild-type, B	26[a]	2.3[a]	0.9[a]
	LBE2045	54	2.8	0.5
	D2216	21[a]	2.6[a]	1.2[a]

[a] These values were taken from [12] and [13]; experimental conditions were slightly different

The apparent equilibrium constant was derived from the two rate constants

It seems to be inherent in the method of determination of the association rate constants of EF-Tu and GTP that one finds values within a variation by a factor of 2–3 (O. Fasano and G. W. M. Swart, unpublished); the values presented here are averages in all 3 cases. Other rate constants are determined very reproducibly

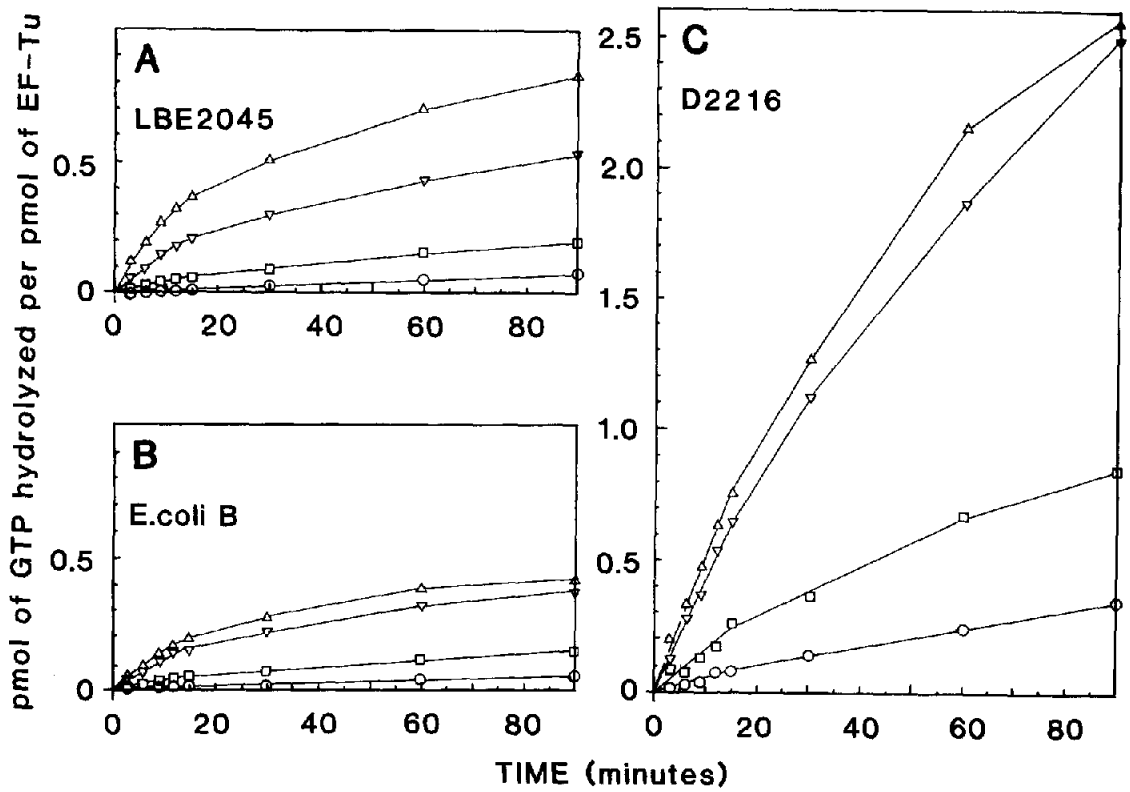

Fig.1. GTPase activity of the pre-formed EF-Tu–GTP complex from strains LBE2045 (A), B (B) and D2216 (C) at 0 (○), 40 (□), 200 (▽) and 400 (△) mM of NH_4^+ at 37°C. The reaction mixtures (0.5 ml) contained 50 mM imidazole acetate (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol and 600 pmol [γ-^{32}P]GTP (~1000 cpm/pmol). Nucleotide-free EF-Tu was added in 150 μl 25 mM imidazole acetate (pH 7.5) and 0.2 mM EDTA. The complex was formed at 0°C in the absence of [NH_4^+] during 15 min. At time 0 [NH_4^+] was adjusted to the desired value and the temperature was raised to 37°C: (A) $EF\text{-}Tu_{LBE2045}$, 140 pmol present, initial complex formation was 80%; (B) $EF\text{-}Tu_B$, 165 pmol present, initial complex formation was 70%; (C) $EF\text{-}Tu_{D2216}$, 75 pmol present, initial complex formation was 75%.

Table 3

Dependence on the [NH_4^+] concentration of the apparent rate constants of the GTPase (A) and dissociation (B) reactions of the EF-Tu–GTP complex from various *E. coli* strains

[NH_4^+] (mM)	Rate constants			Ratio of rate constants	
	Wild-type	LBE2045	D2216	LBE2045 / Wild-type	D2216 / Wild-type
(A) GTPase: EF-Tu–GTP → EF-Tu–GDP + P_i; rate constants in (10^{-4} min^{-1}); temp. 37°C					
0	14	14	75	1.00	5.36
40	49	55	222	1.12	4.51
200	172	207	703	1.20	4.08
400	220	425	997	1.93	4.53
(B) Dissociation: EF-Tu–*GTP + GTP → EF–Tu–GTP + *GTP; rate constants in (10^{-4} s^{-1}); temp. 5°C					
0	187	147	38.5	0.78	0.21
50	154	115	18.2	0.75	0.12
400	27.1	17.5	3.1	0.64	0.11
800	13.7	10.6	1.9	0.77	0.14

Substitution of Ala-375 (R = amino acid side chain = $-CH_3$) by Val ($R = -CH(CH_3)_2$) causes a more drastic change than that by Thr ($R = -CH(OH)CH_3$), since $EF\text{-}Tu_{D2216}$ is more resistant to kirromycin (table 1) it has a higher affinity for GTP (tables 2,3B and it exhibits a stronger activation of its GTPase centre (fig.1, table 3A) than $EF\text{-}Tu_{LBE2045}$.

It has been argued [15,16] that position 375 is most probably the only site of mutation in $EF\text{-}Tu_{LBE2045}$. The same arguments may be raised for $EF\text{-}Tu_{D2216}$, but we are less sure in the latter case because of the type of mutagenesis applied. This finding that the two mutant species of EF-Tu display behavioral shifts in the same direction supports this view and indicates that it is only the mutated position 375 which is responsible for the modified interactions.

The changes in affinity of the 3 species of EF-Tu towards GTP are only expressed in a lowering of the rate of dissociation of the binary complex EF-Tu · GTP and not in the association rate of protein and nucleotide (table 2). It seems justifiable to examine only the latter reaction when studying the dependence on $[NH_4^+]$ of the affinity between EF-Tu and GTP.

All threee EF-Tu species are highly affected by $[NH_4^+]$ in their interaction with GTP (fig.1, table 3). Substitution of Ala-375 by either Thr or Val affects the rate of dissociation of the binary complex. The relative effect of NH_4^+ in decreasing this rate is the same in both mutants. However, when the activation of the GTPase centre is considered, the effect of the replacement of Ala-375 by Thr is not detectable in the absence of added NH_4^+, but is revealed upon raising the $[NH_4^+]$. By contrast the replacement: Ala-375 → Val activates the GTPase centre at all $[NH_4^+]$ to about the same extent.

These and other (P. H. van der Meide, personal communication) data show that the mutation Ala-375 → Thr affects many of the EF-Tu activities, which is also true for kirromycin. Nothing is known so far concerning the binding site of this antibiotic, but kirromycin and GTP do not compete for the same site [11] and moreover amino acid 375 is not located in the nucleotide binding region (T. F. M. LaCour and B. F. M. Clark, personal communication). We suggest that the mutation site is involved in the binding of the antibiotic and that the region around this site is very important in the allosteric regulation of the EF-Tu activities. Two of these activities, binding of GTP (especially the lowering of its dissociation velocity) and hydrolysis of GTP are very closely related, although they seem to be independently regulated. Finally kirromycin greatly amplifies the effect of increasing $[NH_4^+]$ on the interaction between EF-Tu and GTP [14,19].

The strong activation of the GTPase centre which occurs upon substitution of Ala-375 by Val and which is already apparent in the absence of added NH_4^+, may be ascribed to a steric effect of the Val sidechain. Apparently the consequences of Ala-375 → Thr are less extensive since activation of the GTPase centre by the mutation becomes only apparent upon raising the $[NH_4^+]$. Possibly the interaction of the hydroxyl group of threonine with its surroundings may limit the conformational change resulting from the steric effect of the threonine sidechain. When the $[NH_4^+]$ is raised, the allosteric site in $EF\text{-}Tu_{LBE2045}$ is more influenced by these ions and the protein takes on a conformation more similar to that of $EF\text{-}Tu_{D2216}$, as is reflected in the apparent activation of the GTPase centre. However, the nearby allosteric site for regulation of the velocity of EF-Tu–GTP dissociation is protected against an extra influence by increasing $[NH_4^+]$.

Summarizing, we assume that position 375 of the polypeptide chain of EF-Tu is located in an important allosteric region of the molecule. Because of mutation, after binding of kirromycin or under increasing $[NH_4^+]$ the conformation of this site changes such that the affinity for GTP increases and the GTPase centre is activated, probably in an allosteric way.

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