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# Kirromycin-induced modifications facilitate the separation of EF-Tu species and reveal intermolecular interactions

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A simplified method for the separation of a kirromycin-sensitive *tufB*-encoded elongation factor Tu (EF-TuBs) from a kirromycin-resistant *tufA* product (EF-TuAr) was obtained by exploiting the specific increase of positive charges induced by the antibiotic, resulting in a retarded elution of kirromycin-bound EF-TuBs on ionic chromatography. The kirromycin-free EF-TuBs is active in poly(Phe) synthesis and shows similar properties to EF-TuAsBs. As expected for these two distinct species, the dissociation of the EF-TuArBs·GTP complex in the presence of kirromycin shows a biphasic curve; in contrast, a monophasic GTP dissociation rate was found for a combination of two mutated EF-Tu species, EF-TuArBo, revealing the existence of intermolecular interactions. These observations prove for the first time the existence of cooperative phenomena between EF-Tu species in vitro, as suggested earlier by in vivo experiments.

Protein synthesis; Elongation factor Tu; Kirromycin

## 1. INTRODUCTION

EF-Tu is the only GTP binding protein, together with Ha-ras p21, with known three-dimensional structure [1–4] and one of the best characterized members of this class of proteins [5–8]. It has been the object of intensive mutagenesis by diverse procedures (for references, see [9]). The production and isolation of mutated EF-Tu species from *E. coli* in sufficient amounts for biochemical and physico-chemical analysis have been hampered by the presence of large amounts (5–10% of the total cellular proteins) of viable, chromosomal *tuf* gene product essential for cell growth. To overcome this crucial problem, a few years ago a procedure was developed to separate two EF-Tu species by exploiting the differences in affinity for the antibiotic kirromycin and the competition between EF-Ts and kirromycin for binding to EF-Tu [10]. In this method a mixture of kirromycin-resistant and kirromycin-sensitive EF-Tu species, respectively encoded by *tufA* (EF-TuAr) and *tufB* (EF-TuBo) both in complex with EF-Ts, was incubated with kirromycin. The resulting EF-TuAr·EF-Ts and EF-TuBo·kirromycin eluted as 2 separate peaks on ionic chromatography, the complex of EF-TuAr with EF-Ts emerging at much lower salt concentration. This method was also succesfully applied for the separation of a mutated plasmid-borne EF-Tu from cells harbouring only one chromosomal *tuf* gene encoding an EF-TuAr with low affinity for the antibiotic [11].

In this work we show that similar results can be obtained by adding the antibiotic to a mixture of two EF-Tu·GDP species with different affinities to the antibiotic, even in the absence of EF-Ts, as a result of the specific conformational modifications induced by the antibiotic, affecting the net charge of EF-Tu. With this modified method we have separated EF-TuBs from EF-TuAr and compared for the first time the properties of the former with wild-type EF-Tu (EF-TuAsBs). In the course of this study, by measuring the dissociation rates of the GTP complexes of EF-TuAsBs, EF-TuArBs and EF-TuArBo in the presence and absence of the antibiotic, we observed that EF-TuArBo displays in vitro cooperative phenomena, differently from EF-TuArBs. This striking property is discussed in the context of several observations from other laboratories supporting the possibility of cooperative phenomena between EF-Tu mutants in vivo [12–15].

## 2. MATERIALS AND METHODS

The mixture of kirromycin-resistant and kirromycin-sensitive EF-Tu (EF-TuArBs) was extracted by sonication from strain PM816, an *E. coli* K12 strain (obtained from Dr L. Bosch, University of Leiden) harbouring a *tufA* gene encoding kirromycin-resistant EF-TuAr (=EF-TuA375T) and a *tufB* gene encoding kirromycin-sensitive EF-TuBs [16]. EF-TuAr and wild-type EF-Tu (EF-TuAsBs) or the combination of the mutated EF-Tu species (EF-TuArBo) were purified on DEAE-Sepharose Fast Flow (Pharmacia) essentially as reported [17]. EF-TuBo (=EF-TuG222D) was isolated according to [10]. Poly(U)-directed poly(Phe) synthesis and EF-Tu·nucleotide dissociation rates were determined as described [10,18]. Protein concentrations were measured by the method of Bradford [19] using bovine serum al-

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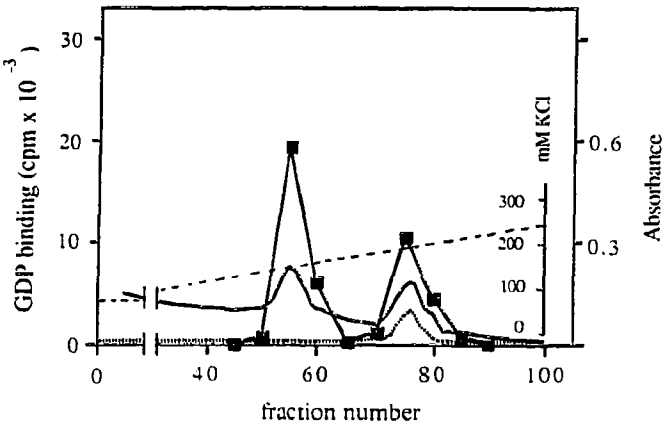


Fig. 1. Separation of EF-TuBs·GDP·kirromycin from EF-TuAr·GDP. A mixture of EF-TuAr·GDP and EF-TuBs·GDP (total EF-Tu concentration 15 $\mu$M) was incubated in buffer A containing 30 mM KCl, 1 mM phenylmethylsulphonyl fluoride and 20 $\mu$M kirromycin for 30 min at 4°C. The incubation mixture was applied to a DEAE Sepharose column (60 × 0.9 cm) and the proteins were eluted with a linear gradient of KCl (dashed line) in buffer A. Fractions were analysed for GDP-binding activity (■) and for the presence of kirromycin by measuring the absorbance at 325 nm (...); (—) absorbance at 280 nm.

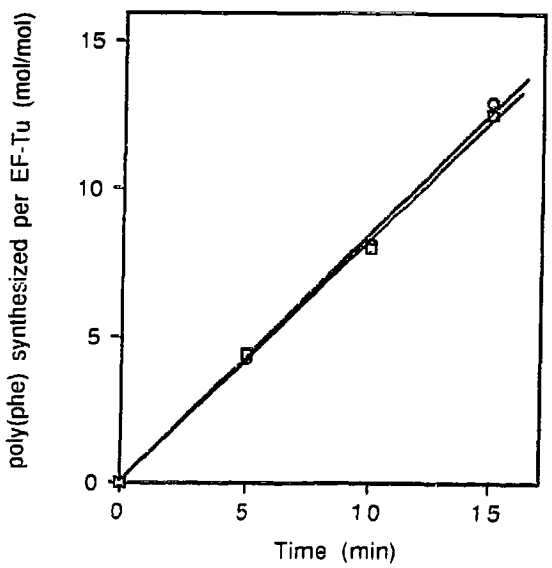


Fig. 2. Poly(U)-directed poly(Phe) synthesis by EF-TuAsBs and EF-TuBs. Poly(U)-directed poly(Phe) synthesis was carried out as reported in [10] with EF-TuAsBs (□) or EF-TuBs (○).

bumine as a standard. The standard assay buffer contained 50 mM Tris-HCl, pH 7.6, 60 mM $NH_4Cl$, 7 mM $MgCl_2$ and 7 mM 2-mercaptoethanol. Buffer A contained 50 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$ and 7 mM 2-mercaptoethanol.

## 3. RESULTS

### 3.1. *Kirromycin-bound form of EF-TuB·GDP is strongly retarded on Ionic Chromatography*

Recently, we have observed that kirromycin strongly modifies the electrophoretic behaviour of EF-Tu, increasing the positive charge as compared to the wild-type [20]. This has led us to test whether the antibiotic alone induces a conformational transition whose charge alteration is sufficient to separate a mixture of two EF-Tu species, of which only one is sensitive to the antibiotic. To this purpose we have partially purified EF-Tu from *E. coli* strain PM816, containing a mixture of EF-TuAr·GDP and wild-type EF-TuBs·GDP. After sonication of the cells and a centrifugation at 30 000 × *g* for 40 min, the cell extract was applied to a DEAE-Sepharose Fast Flow column and eluted with a KCl gradient (50 to 250 mM) in buffer A. The isolated EF-Tu·GDP was more than 60% pure, and the recovery was at least 90%. This EF-Tu·GDP was incubated with kirromycin for 30 min at 4°C, as described in the legend to Fig. 1. The concentration of EF-Tu and kirromycin was about 15 $\mu$M and 20 $\mu$M, respectively, to avoid any binding of the antibiotic to the kirromycin-resistant EF-Tu species. In the following DEAE-Sepharose Fast Flow chromatography (a gradient of 80–250 mM KCl in buffer A) EF-TuAr·GDP and EF-TuBs·GDP·kirromycin were eluted without any overlapping as 2 independent peaks, as a consequence of the strong retardation induced upon EF-TuBs·GDP by the antibiotic (Fig. 1). To obtain the EF-TuBs·GDP free from kirromycin the usual procedure was applied, i.e. a prolonged dialysis in the presence of EF-Ts, exploiting the competition between the latter factor and the antibiotic for binding to EF-Tu [10]. All the steps needed for the separation and isolation of EF-TuBs in its GDP-bound form are resumed in Table I.

Table I

Schematical overview of the procedure for the purification of EF-TuBs

| Purification step | Starting material |
|---|---|
| 1. DEAE Sepharose | Crude cell extract of strain PM816 |
| 2. Kirromycin treatment + DEAE Sepharose | Mixture EF-TuAr·GDP/EF-TuBs·GDP |
| 3. Dialysis in the presence of EF-Ts + DEAE Sepharose | EF-TuBs·kirromycin |
| 4. Incubation with excess of GDP + DEAE Sepharose to replace EF-Ts and to obtain EF-TuBs·GDP | EF-TuBs·EF-Ts |

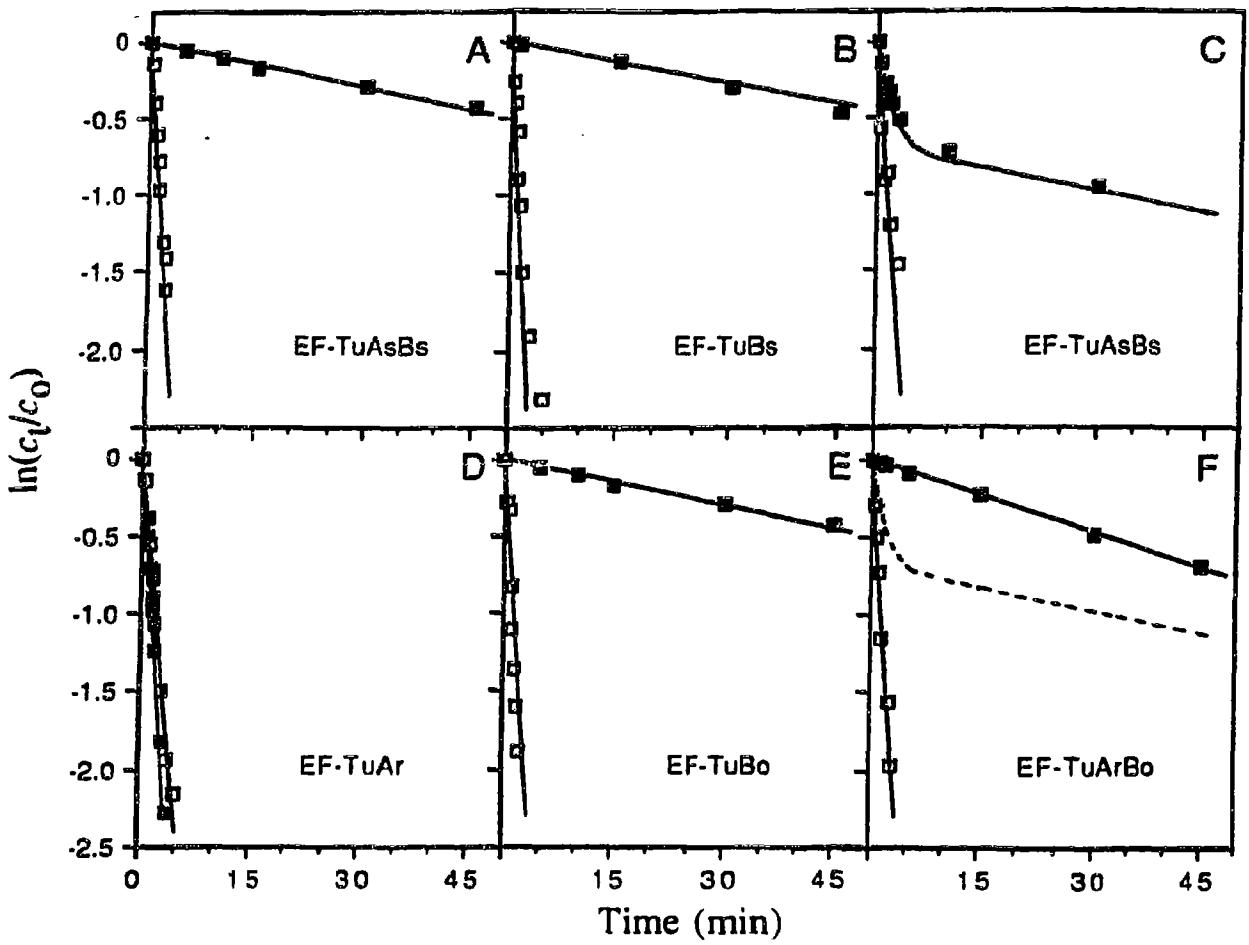


Fig. 3. Effect of kirromycin on the GTP dissociation rates of various EF-Tu species, alone or in combination. Reactions were carried out as described [18]. Nucleotide free EF-Tu (1 $\mu$M) and [$\gamma$-$^{32}$P]GTP (2.5 $\mu$M) were incubated in standard buffer at 0°C for 15 min in the absence (□) or presence (■) of kirromycin (2.5 $\mu$M). The dissociation reaction was started by the addition of 1000-fold excess of unlabeled GTP. At the indicated times, samples were withdrawn and filtered through nitrocellulose filters (Sartorius 0.45 $\mu$m) which were washed twice with cold standard buffer. $\ln(c_t/c_0)=-k_{-1}\cdot t$ were $k_{-1}$ is the dissociation rate constant, $c_0$ is the initial concentration of the EF-Tu·GTP complex and $c_t$ the concentration at different times $t$. In panel F, the broken line represents the theoretical course of the dissociation of a mixture of EF-TuAr·GTP (dissociation half-life 70 s) and EF-TuBo·GTP·kirromycin (dissociation half-life 75 min).

The isolation of EF-TuBs in homogeneous form enabled us to test whether the purified *tufB* product, which differs only at its C-terminus from the *tufA* gene product (Ser instead of Gly), would display any functional differences from wild-type EF-Tu which is constituted in a 60 to 40% ratio by the *tufA* and *tufB* products [16] (see Fig. 1). In Fig. 2 is shown that EF-TuBs is as active in poly(UP)-directed poly(Phe) synthesis as EF-TuAsBs. Its sensitivity to kirromycin is also similar to that of EF-TuAsBs (not shown). The dissociation of the respective complexes with GDP and GTP, parameters which are very sensitive to functional modifications, occurs with comparable half-life values (see below). These results strongly suggest that the activity of EF-TuBs is identical to that of EF-TuAsBs and as a consequence as that of EF-TuAs.

### 3.2. *Intermolecular Interactions of EF-Tu*

During the isolation and characterization of the different species of EF-Tu we have met with some observations on EF-TuArBo from strain LBE2012, that could only be explained by assuming the occurrence of intermolecular interactions of EF-Tu and, thus, could support the possibility of reported cooperative phenomena in vivo ([12–15]; see also section 4). As a relevant background to our in vitro experiments, one should remember that the interaction of EF-Tu with guanine nucleotides is characterized by a difference of 2 orders of magnitude in the binding for GDP and GTP. The main cause for this difference is the slower dissociation rate of GDP from EF-Tu·GDP as compared to the faster dissociation rate of GTP from EF-Tu·GTP. In the presence of kirromycin the dissociation rate of GTP from the EF-Tu·GTP·kirromycin complex is drastically reduced so that the affinity of EF-Tu for GTP becomes of the same order as that for GDP [18]. Fig. 3A, B and E illustrate this phenomenon for several kirromycin-sensitive EF-Tu preparations (half-life 70 s for EF-

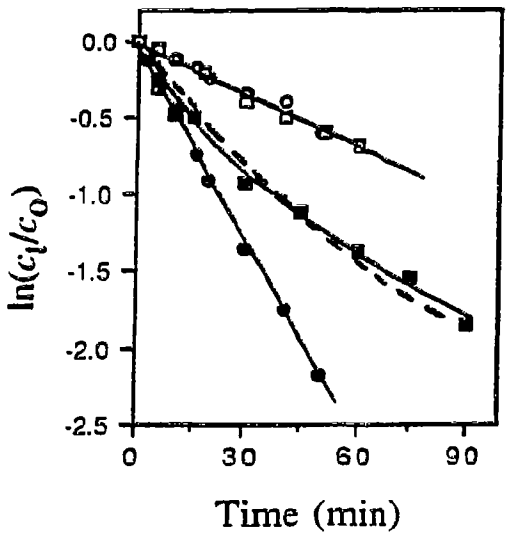


Fig. 4. Effect of kirromycin on the GDP dissociation rates of EF-TuBs and EF-TuArBo. Reactions were carried out as described in the legend of Fig. 3, except that [$^3$H]GDP replaced [$\gamma$-$^{32}$P]GTP. EF-Tu (1 $\mu$M, nucleotide free) and [$^3$H]GDP (1 $\mu$M) were incubated at 0°C for 15 min in standard buffer in the absence (open symbols) or presence (closed symbols) of 2.5 $\mu$M kirromycin. Reactions were started by the addition of 1000-fold excess of unlabeled GDP. (○,●) EF-TuBs; (□,■) EF-TuArBo; the broken line represents the theoretical course of dissociation of an equimolar mixture of EF-TuAr·GDP (dissociation half-life 50 min) and EF-TuBo·GDP·kirromycin (dissociation half-life 15 min).

TuAsBs and EF-TuBs and 51 s for EF-TuBo in the absence, versus 75 min in the presence of kirromycin), while Fig. 3D shows that addition of the antibiotic hardly affects the behaviour of kirromycin-resistant EF-TuAr (half-life 70 versus 75 s). Taking into account these different GTP dissociation properties of EF-Tu·GTP and EF-Tu·GTP·kirromycin one should be able to reveal a heterogeneity in mixed factor preparations containing both kirromycin-sensitive and kirromycin-resistant EF-Tu upon addition of a limiting amount of the antibiotic. Indeed, this applies to the mixture EF-TuArBs (Fig. 3C): EF-TuAr·GTP displays a fast dissociation (half-life 70 s), while EF-TuBs·GTP·kirromycin forms a stable complex (half-life 75 min). Most strikingly however, no trace of a fast component is apparent in the dissociation of EF-TuArBo·GTP·kirromycin; instead, one observes a monophasic dissociation with a relatively long half-life (45 min; Fig. 3F), which is in the range of that of kirromycin-sensitive EF-Tu. In an attempt to explain this phenomenon one has to assume an intermolecular interaction between the 2 mutant EF-Tu species, causing an altered behaviour of EF-TuAr towards the antibiotic.

We tested whether these effects occurred for the GDP-bound forms of EF-TuArBo. It is known, that the dissociation rates of EF-TuAsBs·GDP [18], EF-TuBo·GDP [10] or EF-TuBs·GDP (Fig. 4) are a few times faster in the presence of kirromycin (half-life 50 min in the absence and 15 min in the presence of the antibiotic), while that of EF-TuAr·GDP is unaffected. As shown in Fig. 4, differently from the results obtained with the corresponding GTP-bound complex, kirromycin reveals heterogeneity in the dissociation rate of EF-TuArBo in the presence of GDP, suggesting that the cooperative phenomena strictly depend on the GTP-bound form and the presence of a specific mutation in EF-Tu (Gly$^{222}$→Asp). We could not test the behaviour of the mixture of EF-TuAs and EF-TuBo with this method, because these EF-Tu species are both sensitive to kirromycin.

## 4. DISCUSSION

The observation that binding of kirromycin to EF-Tu·GDP induces an alteration of the net charge of the factor to an extent allowing the complete separation of the EF-Tu·GDP·kirromycin complex from EF-TuAr·GDP on ionic chromatography, simplifies the procedure for isolating EF-Tu mutants overproduced in *E. coli*. Moreover, it shows how deeply the selective action of kirromycin modifies the EF-Tu·GDP conformation. The advantage of using EF-Tu·GDP instead of EF-Tu·EF-Ts is evident. EF-Tu·GDP, which accounts for 80–90% of the total EF-Tu in the cell, can be easily obtained from the cell extract up to 60–80% pure in a one-step separation either by ionic or affinity chromatography. Thus the largest part of EF-Tu may be straigtly processed as EF-Tu·GDP complex, markedly increasing the yield of the mutated protein. We have succesfully applied this method not only to the separation of the *tufB* product as described in this paper, but also of the overproduced EF-TuC81G, a factor mutated in a residue located in the guanine nucleotide binding site (P.H. Anborgh and J. Jonak, unpublished results). The characterization of EF-TuBs in pure form has led us to the conclusion that no functional difference exists between the *tufB* gene product and wild-type EF-Tu, as also suggested by Vijgenboom [21], who separated the EF-TuBs factor using another method.

Of particular interest is the observation that 2 mutated EF-Tu species (EF-TuAr and EF-TuBo) show intermolecular interactions in an in vitro system, the kirromycin-sensitive EF-Tu being dominant over the kirromycin-resistant EF-Tu. Cooperative effects of these two EF-Tu species have already been reported in vivo. Vijgenboom et al. [12] observed that the suppression of nonsense codons requires the combined action of EF-TuAr and EF-TuBo, the combinations EF-TuAr with EF-TuBs or EF-TuAs with EF-TuBo being ineffective. In line with this, Hughes [13] reported that in vivo suppression of certain termination codons in *Salmonella typhimurium* is much more efficient if both *tuf* gene products are mutated. Strains with a mutation in both *tuf* genes are particularly active in suppressing −1/+1 frameshifting by EF-Tu [14,15]. These reports fit

well with our observations in vitro and suggest that intermolecular interactions in EF-Tu are related to specific substitutions. However, one should mention that other authors contest the importance of EF-TuBo for the suppression of nonsense codons by EF-TuAr [22]. Nevertheless, the possibility of the participation of 2 molecules of EF-Tu·GTP in the formation of a pentameric complex with aa-tRNA [23] and the observation by small angle neutron scattering of the presence of EF-Tu dimers in solution [24] appears to extent the importance of intermolecular interactions also to the activity of wild-type EF-Tu, underlining the need for further studies aimed at clarifying the functional aspects of these phenomena.

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