Kirromycin-resistant elongation factor Tu from wild-type of *Lactobacillus brevis*

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Properties of the elongation factor Tu from *Lactobacillus brevis* which is naturally insensitive to kirromycin are described. The protein is characterized by an unusual nucleotide-binding site with increased affinity for GTP and extreme heat stability. EF-Tu is sensitive to pulvomycin in the assay of polyphenylalanine synthesis. However, the failure of the protein to display pulvomycin-dependent GDP-binding and GTPase activity indicates that pulvomycin action in *L. brevis* differs from that in *E. coli*.

Elongation factor Tu Lactobacillus brevis *Kirromycin resistance* *Pulvomycin*
Nucleotide-binding site *Heat stability*

1. INTRODUCTION

The antibiotics, kirromycin and pulvomycin, inhibit prokaryotic protein synthesis by interaction with the elongation factor Tu [1,2]. Mutants of *Escherichia coli* and *Bacillus subtilis* selected for resistance to kirromycin have been shown to possess an altered form of EF-Tu [3–5]. Acquisition of kirromycin resistance introduces functionally related modifications of the EF-Tu nucleotide-binding site [3,4]. Here, we describe properties of EF-Tu from *Lactobacillus brevis* which is naturally insensitive to kirromycin. This protein is characterized by an unusual nucleotide-binding site with increased affinity for GTP and extreme heat stability.

2. MATERIALS AND METHODS

Elongation factors and ribosomes of *E. coli* MRE 600 were prepared as in [6,7]. EF-Tu of *L. brevis* DSM 20,054 was isolated from cell extracts by chromatography on DEAE–Sepharose, phenyl–Sepharose, and Sephadex G-100. Wet cells (100 g) yielded 70 mg homogeneous EF-Tu. The M_r of EF-Tu (Lb) was determined by SDS–polyacrylamide gel electrophoresis to be 51 000. Nucleotide-free EF-Tu was prepared according to [8]. Kirromycin and pulvomycin were prepared as in [9,10]. Interaction of EF-Tu with nucleotides, Phe-tRNA and EF-Ts was measured as in [8]. Polyphenylalanine synthesis was performed in the *E. coli* system [2] replacing, where indicated, EF-Tu (Ec) by the corresponding protein of *L. brevis*.

Abbreviations: EF, elongation factor; EF-Tu$_f$, nucleotide-free elongation factor Tu; affices refer to the source of the protein, Ec, *Escherichia coli*; Lb, *Lactobacillus brevis*

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3. RESULTS AND DISCUSSION

3.1. *Interaction of EF-Tu with kirromycin and pulvomycin*

Fig.1 shows that both antibiotics inhibited polyphenylalanine synthesis in a cell-free system of *E. coli*. When in this system EF-Tu (Ec) was replaced by the corresponding protein of *L. brevis*, the concentration, needed for 50% inhibition, was 500-fold higher. As for pulvomycin, differences in the sensitivity are less pronounced; polyphenylalanine synthesis catalyzed by EF-Tu (Lb) was 3–5-times less susceptible to inhibition by this drug than the same reaction catalyzed by EF-Tu (Ec).

The ability of either antibiotic to stimulate the exchange of EF-Tu-bound GDP with unbound GDP at 0°C [1,2] was used as another test for the

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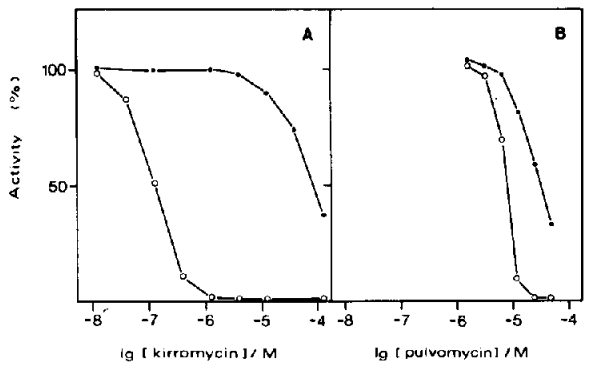

Fig.1. Inhibition of polyphenylalanine synthesis catalyzed by (•) EF-Tu (Lb) or (○) EF-Tu (Ec) as a function of antibiotic concentration. Reaction mixtures contained, in 100 μl standard buffer (60 mM Tris • HCl (pH 7.6), 30 mM KCl, 30 mM NH_4Cl, 10.5 mM $MgCl_2$, 0.5 mM EDTA, 5 mM mercaptoethanol), 100 nmol GTP, 100 nmol phosphoenolpyruvate, 2 μg pyruvate kinase, 2.5 pmol ribosomes (Ec), 1 μg poly (U), 24 pmol EF-G(Ec), 35 pmol [^{14}C]Phe-tRNA, 30 pmol EF-Tu (Lb) or (Ec), and 1 μl methanol solution of antibiotic as indicated. Samples were incubated for 10 min at 30°C and then the radioactivity incorporated into hot trichloroacetic acid-insoluble protein was measured.

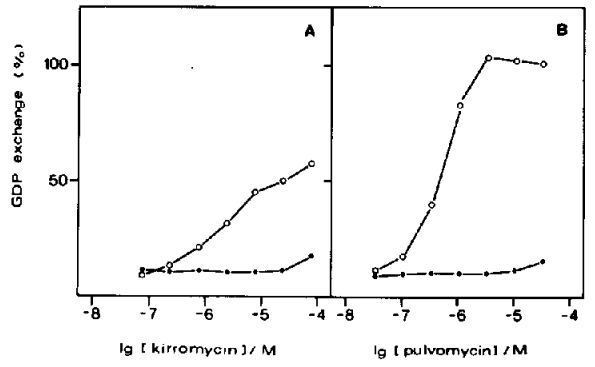

Fig.2. Binding of GDP by (•) EF-Tu (Lb) or (○) EF-Tu (Ec) as a function of antibiotic concentration. Reaction mixtures contained, in 150 μl standard buffer, 50 pmol EF-Tu • GDP, 150 pmol [^{3}H]GDP and 1 μl methanol solution of antibiotic as indicated. Following a 5 min incubation at 4°C, samples were analyzed for [^{3}H]GDP reatined on cellulose nitrate filters. Data are presented as percentage of GDP exchange after incubation for 10 min at 30°C in the absence of antibiotics.

interaction of the drugs with EF-Tu (Lb). Kirromycin and pulvomycin were found to stimulate strongly the nucleotide-binding activity of EF-Tu (Ec) (fig.2). When EF-Tu (Lb) was used in this assay, the 2 antibiotics virtually do not affect the exchange reaction. These results indicate that EF-Tu (Lb) is insensitive to both antibiotics.

Similar results were obtained by measuring the EF-Tu GTPase activity. The hydrolysis of GTP involved in the binding of AA-tRNA to ribosomes requires EF-Tu, AA-tRNA, ribosomes and mRNA [8]. In the presence of both pulvomycin or kirromycin, EF-Tu can catalyze the reaction in the absence of the other components [1,2]. When EF-Tu of *L. brevis* was used in the antibiotic-dependent hydrolysis of GTP, the two drugs failed to induce GTPase activity even at 100 μM (not shown).

Binding of the antibiotics to EF-Tu (Lb) was studied by equilibrium gel permeation [11]. Elution profiles of the 313-nm absorbance during the passage of the protein through a Sephadex G-25 column, equilibrated with kirromycin (a_m 28 000) or

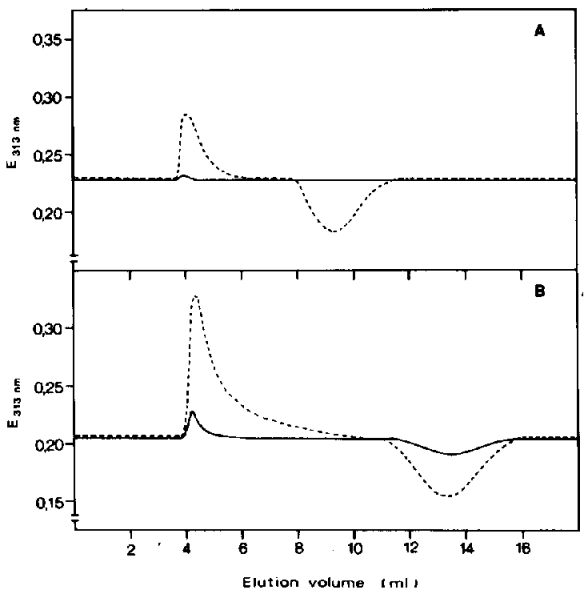

Fig.3. Binding of (A) kirromycin and (B) pulvomycin to EF-Tu. Elution profile of the 313-nm absorbance during the passage of (——) 7.2 nmol of EF-Tu (Lb) or (- - -) 2.4 nmol of EF-Tu (Ec) through a column (39 × 0.6 cm) of Sephadex G-25 fine, equilibrated with 8.2 μM kirromycin or 5.4 μM pulvomycin in standard buffer. Flow rate 6 ml/h; temp. 20°C.

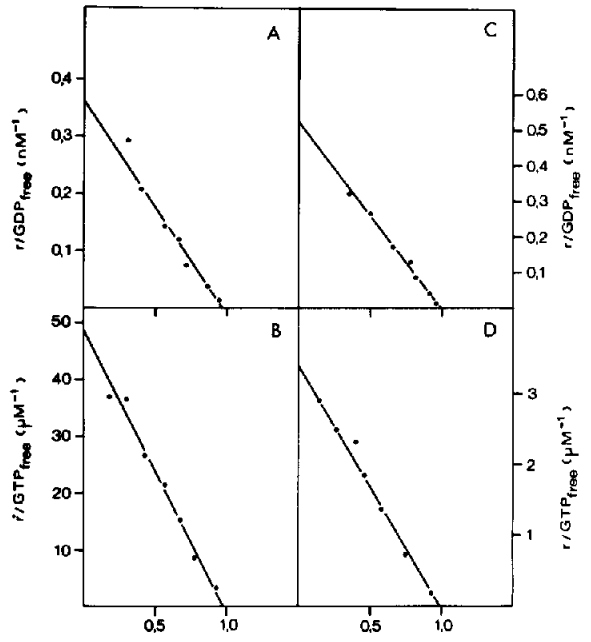

Fig.4. Scatchard plots for nucleotide binding to EF-Tu: (A) binding of GDP to EF-Tu (Lb); (B) binding of GTP to EF-Tu (Lb); (C) binding of GDP to EF-Tu (Ec); (D) binding of GTP to Ef-Tu (Ec). Reaction mixtures contained, in 150 μl standard buffer, 5 nM EF-Tu$_f$ and 2.6–65 nM [^{3}H]GDP, or 30 nM EF-Tu (Lb)$_f$ and 12–300 nM [^{3}H]GTP, or 300 nM EF-Tu (Ec)$_f$ and 120–3000 nM [^{3}H]GTP. Following a 10 min incubation at 30°C, samples were analyzed for [^{3}H]nucleotide retained on cellulose nitrate filters. r is the concentration of bound nucleotide divided by the total concentration of EF-Tu$_f$.

pulvomycin (a_m 38 000), are shown in fig.3. Both the void volume peak and the trough observed on the absorbance baseline provide a measure of the drug bound to the protein. When EF-Tu (Lb) was applied to the column, low affinity interaction was demonstrated by the presence of a small peak and a small trough. Virtually no interaction was detected between kirromycin and EF-Tu (Lb). The minor peak (fig.3A) was caused by free protein. Thus, the difference in responsiveness of EF-Tu (Lb) to both antibiotics observed in the polyphenylalanine synthesis is due to a difference in affinity. In contrast, EF-Tu (Ec) bound both kirromycin and pulvomycin with high affinity.

3.2. *Dissociation constants of EF-Tu · GDP and EF-Tu · GTP*

Dissociation constants of EF-Tu · GDP and EF-Tu · GTP from *L. brevis* were calculated from Scatchard plots (fig.4A,B) to be 3 nM and 20 nM, respectively. For comparison, dissociation constants of EF-Tu · GDP and EF-Tu · GTP from *E. coli* were determined to be 2 nM and 300 nM, respectively (fig.4C,D). The latter data are similar to those in [12]. While the affinity for GDP is nearly the same for EF-Tu from both organisms, EF-Tu (Lb) has a considerably higher affinity for GTP compared to EF-Tu (Ec).

3.3. *Heat stability*

EF-Tu of *E. coli* is known to be highly sensitive to thermal inactivation [13]. This protein heated at 60°C for 5 min lost its ability to bind GDP (fig.5A). By contrast, EF-Tu of *L. brevis* withstands heating even at just below 100°C. EF-Tu (Lb) heated at 90°C for the same time retained 90% of its nucleotide-binding capacity (fig.5). GDP is not needed for protection as the same results were obtained with EF-Tu (Lb)$_f$ in the absence of GDP in the incubation mixture. However, heat stability of EF-Tu (Lb) is severely restricted to nucleotide-binding. Concerning other functional parameters (catalyzing polyphenylalanine synthesis, binding of AA-tRNA, interaction with EF-Ts), the protein of *L. brevis* and *E. coli* were inactivated at equal temperatures (fig.5B,D).

These results show that EF-Tu (Lb) is resistant to kirromycin by virtue of its inability to bind this antibiotic. On the other hand, EF-Tu (Lb) is sensitive to pulvomycin in the assay of polyphenylalanine synthesis. However, the failure of this protein to display pulvomycin-dependent GDP-binding and GTPase activity indicates that pulvomycin action in *L. brevis* differs from that in *E. coli*. Recently, EF-Tu factors of *Halobacterium cutirubrum* [14] and *Streptoverticillium mobaraense* [15] have been found to be also naturally resistant to kirromycin.

In addition to kirromycin resistance, the protein of *L. brevis* is characterized by an unusual nucleotide-binding site. EF-Tu (Lb) has a considerably higher affinity than EF-Tu (Ec) for GTP. In this respect, the protein is similar to that of kirromycin-resistant mutants of *E. coli* [16]. Further more, EF-Tu(Lb) is extremely thermostable;

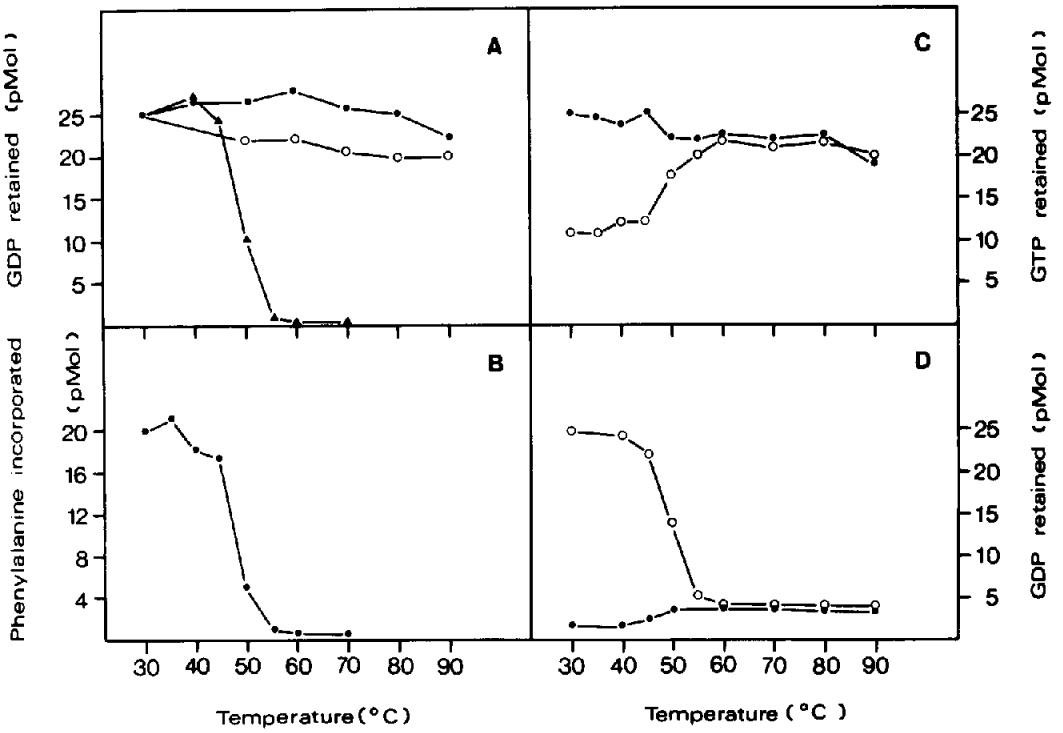

Fig.5. Heat stability of EF-Tu. 30 pmol EF-Tu • GDP and 50 pmol GDP (or 30 pmol EF-Tu$_f$ in the absence of GDP) in 50 μl standard buffer were incubated for 5 min at the indicated temperatures and then assayed for remaining activities: (A) GDP binding activity of (•) EF-Tu (Lb) • GDP, (○) EF-Tu (Lb)$_f$ and (▲) EF-Tu (Ec) • GDP (B) polyphenylalanine synthesis catalyzed by EF-Tu (Lb) • GDP (C) ternary complex formation. After conversion of EF-Tu (Lb) • GDP to EF-Tu (Lb) • GTP, interaction with 50 pmol Phe-tRNA was assayed by the filtration method. The release of bound EF-Tu • GTP from cellulose nitrate filters upon addition of AA-tRNA provides a measure of complex formation. (○) With Phe-tRNA, (•) without Phe-tRNA. (D) Interaction of EF-Tu (Lb) • GDP with EF-Ts. GDP binding of the protein at 4°C was determined in the (○) presence and (•) absence of EF-Ts. EF-Ts is known to catalyze the EF-Tu • GDP/GDP exchange.

however, the heat stability is restricted to the nucleotide-binding site suggesting a rigid structure of this part of the EF-Tu molecule.

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