

# Asparagine-135 of elongation factor Tu is a crucial residue for the folding of the guanine nucleotide binding pocket

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This work studies the structure-function relationships of Asn<sup>135</sup>, a residue situated in the GTP binding pocket of elongation factor Tu (EF-Tu). For this purpose we constructed EF-TuN135D/D138N and assayed its reactivity towards various purine nucleotides. We found that EF-TuN135D/D138N had no functional effect with GTP, ATP, XTP and *iso*GTP. The lack of a productive interaction with *iso*GTP shows that the Asn<sup>135</sup> side-chain does not recognize the exocyclic keto group of the guanine base. However, EF-TuN135D/D138N, whose native conformation is stabilized by either elongation factor Ts or kirromycin, was able to support the enzymatic binding of aa-tRNA to the ribosome in the absence of any nucleotide, when in complex with the antibiotic. Taken together, these results show that Asn<sup>135</sup> is important for the correct folding of the nucleotide binding site and that EF-Tu-kirromycin can mediate the binding of aa-tRNA to the mRNA-programmed ribosomes independently of the native conformation of this site.

EF-Tu; Folding of GTP-binding pocket; Base recognition; *iso*GTP

## 1. INTRODUCTION

A large number of cell pathways are regulated by guanine nucleotide binding proteins [1,2]. So far the three-dimensional structure (3D) of only two of these proteins have been elucidated: elongation factor Tu (EF-Tu), an essential component of bacterial protein biosynthesis, and c-H-ras p21, a regulatory element of growth control and cell division in mammals. As for all GTP-binding proteins, the active form of EF-Tu is the complex with GTP, that is characterized by a high affinity for aa-tRNA and ribosomes, whereas the complex with GDP interacts productively only with elongation factor Ts (EF-Ts). After the hydrolysis of GTP, that follows the interaction between the aa-tRNA·EF-Tu·GTP complex and the mRNA-programmed ribosome, the resulting EF-Tu·GDP leaves the ribosome and is regenerated to the active form via a GDP to GTP exchange reaction specifically stimulated by EF-Ts [3,4]. The affinity of EF-Tu for GTP, which is much lower than that for GDP, is strongly increased by the binding of aa-tRNA [5]. EF-Tu binds guanine nucleotides in a highly specific manner. The replacement of guanine with hypoxanthine or xanthine impairs the binding of the nucleotide by two orders of magnitude; and with adenine or cytosine by four orders [3,6]. The 3D model of a nicked EF-Tu·GDP molecule at medium resolution (2.6 Å) suggests that the aminogroup N(2) of

the base forms a strong hydrogen bond with Asp<sup>138</sup> of EF-Tu [7,8], similarly to the corresponding residue (Asp<sup>119</sup>) in the 3D model of c-H-ras p21 at 1.35 Å resolution [9]. Raman spectroscopy confirmed the existence of this hydrogen bond [10]. Functional data have supported this possibility; in 1987 Hwang and Miller [11] and recently Weijland and Parmeggiani [12] observed that EF-TuD138N recognizes XTP with an affinity comparable to that of wild-type EF-Tu (EF-Tu wt) for GTP. Less clear is the situation concerning the exocyclic O(6) of the base. In 1985 LaCour et al. [7] suggested that the side-chain amino group of Asn<sup>135</sup> could form a hydrogen bond with O(6) of guanine. For Jurnak [8] this residue was situated directly over the plane of the guanine ring but a weak interaction with this base was not excluded. The recently refined model of EF-Tu·GDP [13] describes for the amino side-chain of Asn<sup>135</sup> the hydrogen bonds with O(6) and N(7) but also mentions, as a more probable alternative, a hydrogen bond with the main-chain (MC) carbonyl group of His<sup>22</sup>, a residue that is part of the consensus element <sup>18</sup>GXXXXGK involved in the phosphate binding. The latter situation is similar to the p21 model of Pai et al. [9]. In p21, the guanine O(6) forms a hydrogen bond with the MC carbonyl of Ala<sup>146</sup> (Ala<sup>174</sup> in EF-Tu), excluding a hydrogen bond with Asn<sup>116</sup> (Asn<sup>135</sup> in EF-Tu), a residue that interacts weakly with the N(7) of the base and ties together three fundamental consensus sequence elements (<sup>10</sup>GXXXXGK; <sup>116</sup>NKXD and <sup>145</sup>SAK) essential for a correct architecture of the GTP binding pocket. Cur-

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ously, in a p21-GDP model at 2.5 Å Milburn et al. [14] depict a hydrogen bond between Asn<sup>116</sup> side-chain and O(6) of the base.

In the actual state of our 3D knowledge, substitution of both Asn<sup>135</sup> and Asp<sup>138</sup> would define the role of Asn<sup>135</sup> in the nucleotide binding site of EF-Tu and unequivocally elucidate the participation of the side-chain nitrogen of Asn<sup>135</sup> in the recognition of the base. Indeed, should Asn<sup>135</sup> interact with guanine O(6), EF-TuN135D/D138N would be expected to interact productively with *iso*GTP, an analogue of GTP in which the exocyclic nitrogen and oxygen of the base are substituted by an oxygen and nitrogen, respectively. The observation that EF-TuN135D is as active as EF-Tu wt, if GTP is replaced with XTP [11] supports this assumption. It was moreover interesting to test the ability of EF-TuN135D/D138N to interact with the EF-Tu ligands.

## 2. MATERIALS AND METHODS

EF-TuN135D/D138N was constructed by site-directed mutagenesis of the *tufA* gene cloned in pEMBL9<sup>+</sup>. The oligodeoxynucleotide GTGTCCTGGACAAATG was used to substitute Asn<sup>135</sup> with Asp in EF-TuN138N [12]. The modified *tufA* was overexpressed via pTTQ18 under control of the *tac* promoter in the RecA<sup>+</sup> *E. coli* strain PM1455, containing only one active *tufA* gene encoding a kirromycin-resistant product [15]. EF-Tu N135D/D138N was purified according to the method utilizing the antibiotic kirromycin [12].

The reactivities of EF-Tu wt and N135D/D138N to GTP, ATP, XTP and *iso*GTP or the EF-Tu-dependent binding of Phe-tRNA<sup>Phe</sup>

to the mRNA-programmed ribosome were assayed by retention of the labelled compound on nitrocellulose filters [12]. XTP, GTP, ITP and ATP were obtained from Sigma. The *iso*GTP was synthesized by radiation of ATP with a Hanau Lamp with Heliosil filter,  $\lambda = 245$  nm [17]. Conversion of ATP to *iso*GTP takes place via 1-oxide ATP. In the last step *iso*GTP was purified on a nucleosil 5C18 column using a linear gradient ranging from 5 to 25% acetonitrile in triethylammonium acetate. The composition was analyzed by the absorption spectrum and by <sup>1</sup>H-, <sup>13</sup>C-, and <sup>31</sup>P-NMR. The NMR spectra are shown in Fig. 1. [ $\gamma$ -<sup>32</sup>P] XTP and [ $\gamma$ -<sup>32</sup>P]*iso*GTP were synthesised by the method of Glynn and Chappell [16]. [ $\gamma$ -<sup>32</sup>P]GTP and [ $\gamma$ -<sup>32</sup>P]ATP were from Amersham. [<sup>14</sup>C]Phe-tRNA<sup>Phe</sup> was prepared as reported [18].

The EF-Tu-dependent hydrolysis of the nucleoside triphosphates was measured by the charcoal method and protein concentrations were determined by the Bradford method using bovine serum albumin as a standard [19].

## 3. RESULTS

Fig. 2 shows the structure of the nucleotide bases (adenine, guanine, isoguanine, hypoxanthine and xanthine) used or mentioned in this work and Fig. 3 the interactions involved in the recognition of the exocyclic groups of the base in the case of EF-Tu wt, EF-TuN135D/D138N and EF-TuN135D, as derived from the most recent model of Kjeldgaard and Nyborg [13]. The procedure for the isolation of EF-TuN135D/D138N took into consideration its inability to interact with GTP [12] and the very low concentration, if any, of XTP in the bacterial cell. Nucleotide-free EF-Tu wt is very unstable but it is stabilized by EF-Ts or kirromycin, two ligands behaving as chaperone-like molecules

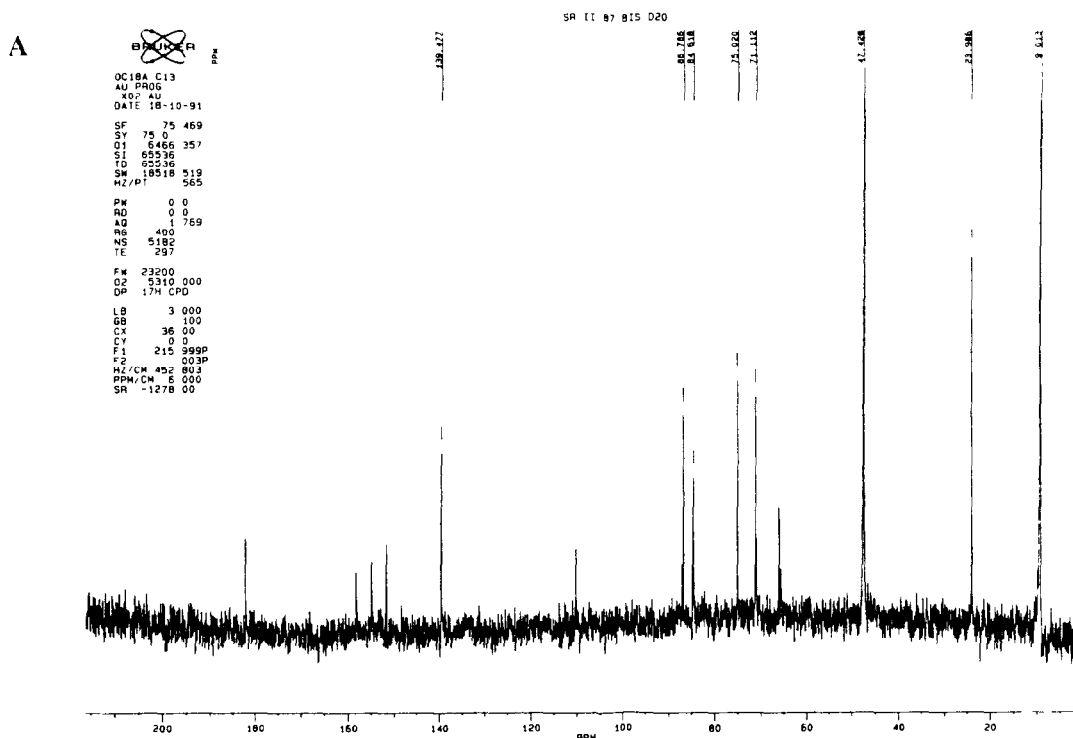


Fig. 1. <sup>13</sup>C-, <sup>1</sup>H-, <sup>31</sup>P-NMR spectra of *iso*GTP. (A) <sup>13</sup>C-NMR 75MHz: (D<sub>2</sub>O)  $\delta = 65.95$ (C-5'); 71.08(C-3'); 75.00(C-2'); 84.59(C-4'); 86.75(C-1'); 110.18(C-5); 139.44(C-8); 151.6(C-4); 154.8(C-6); 158.0(C-2).

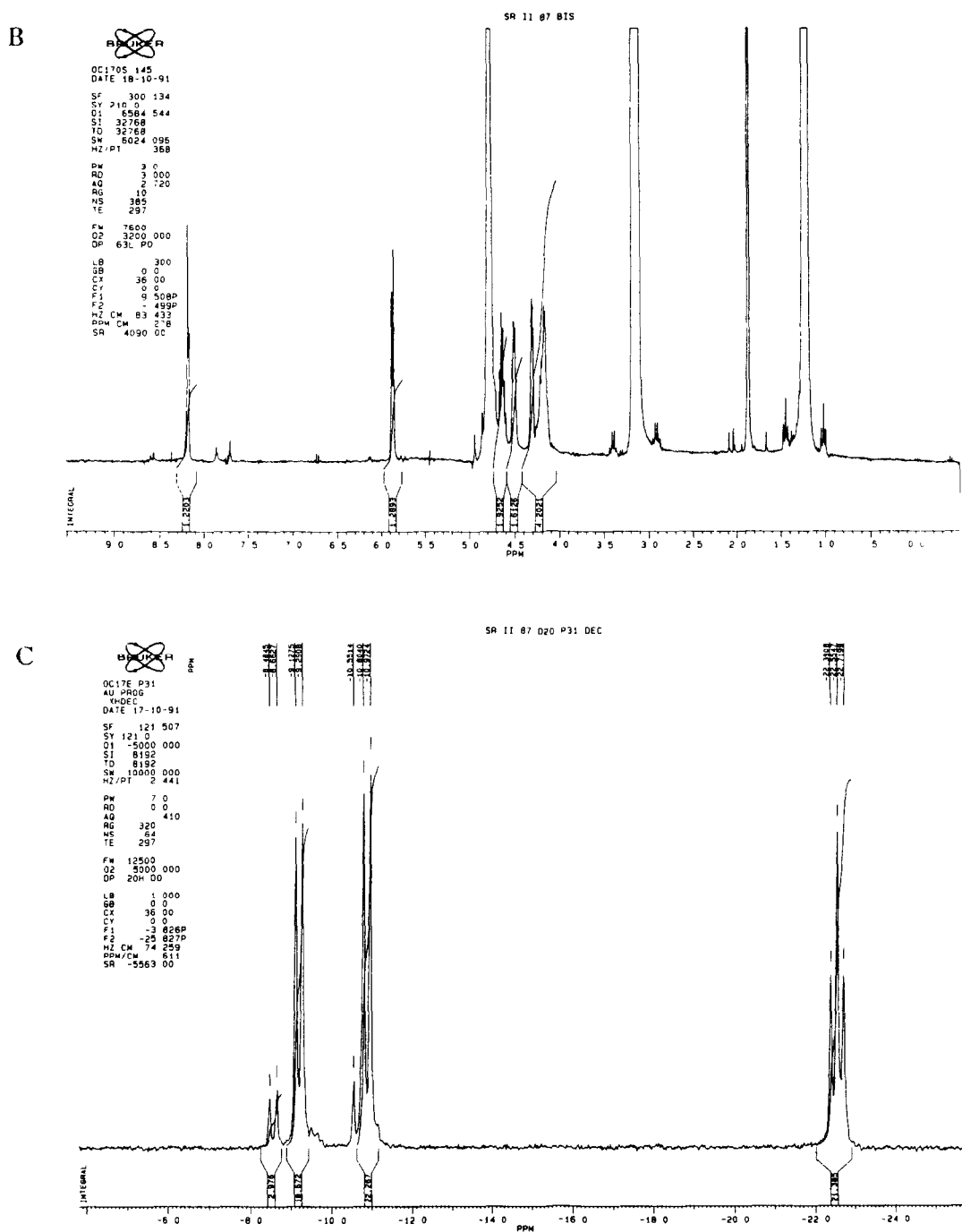


Fig. 1 (continued) (B)  $^1\text{H}$ -NMR 300MHz: ( $\text{D}_2\text{O}$ )  $\delta = 5.87(\text{d}, 1\text{H}, J_{1,2} = 6.5 \text{ Hz}, \text{H-1}), 8.2(\text{s}, 1\text{H}, \text{H-8})$ . (C)  $^{31}\text{P}$ -NMR 121MHz: ( $\text{D}_2\text{O}$ )  $\text{d}(\text{PO}_4\text{H}_2$  as reference) =  $-9.2(\text{d}, 1\text{P}, \text{P}_\gamma, J_{\text{P}_\gamma-\text{P}_\beta} = 20.6 \text{ Hz})$ ;  $-10.89(\text{d}, 1\text{P}, \text{P}_\alpha, J_{\text{P}_\alpha-\text{P}_\beta} = 20.6 \text{ Hz}, J_{\text{P}_\alpha-\text{P}_\gamma} = 0 \text{ Hz})$ ;  $-22.55(\text{d}, 1\text{P}, \text{P}_\beta, J_{\text{P}_\beta-\text{P}_\alpha} = J_{\text{P}_\beta-\text{P}_\gamma} = 20.6 \text{ Hz})$ .

in preserving the active conformation of EF-Tu [20]. Therefore for the purification of EF-TuN135D/D138N we applied the method using EF-Ts and kirromycin [21, 22], that allows the protection of EF-Tu against denaturation from the overproduction in the cell through the purification procedure and its characterization. The soluble plasmid-borne kirromycin-bound EF-TuN135D/D138N was fully separated from the chromosomal kirromycin-resistant EF-TuAr on DEAE chromatogra-

phy, and then freed from the antibiotic by dialysis in the presence of EF-Ts [21, 22]. Kirromycin- or EF-Ts-bound EF-TuN135D/D138N was stable for at least one year when kept at  $-20^\circ\text{C}$  in 50 mM Tris-HCl buffer, pH 7.6, 70 mM  $\text{NH}_4\text{Cl}$ , 7 mM  $\text{MgCl}_2$ , 1 mM dithiothreitol and 50% glycerol. The yield was less than 1 mg per 20 g of cells.

The experimental results showed that neither GTP, ATP, XTP or, most importantly, *iso*GTP, up to 10  $\mu\text{M}$

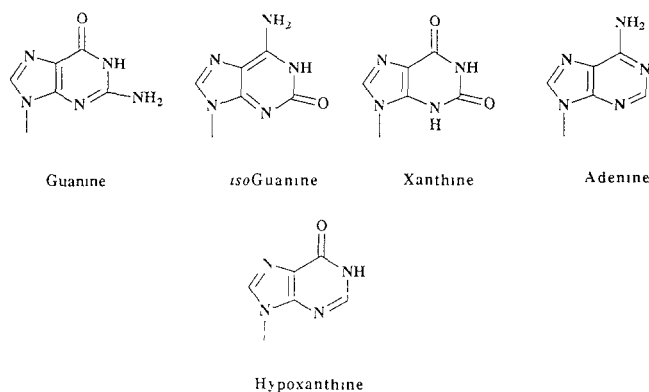


Fig. 2. The structure of the bases of the nucleotides used for this study.

concentration, could be bound to EF-TuN135D/D138N in detectable amounts, even if kirromycin or EF-Ts were present (not illustrated). The former ligand is known to increase the affinity of EF-Tu for GTP by more than two orders of magnitude [5]. In line with these experiments, no hydrolytic activity dependent on EF-TuN135D/D138N could be detected either using [ $\gamma$ - $^{32}$ P]ATP, [ $\gamma$ - $^{32}$ P]XTP, [ $\gamma$ - $^{32}$ P]GTP or [ $\gamma$ - $^{32}$ P]isoGTP, even in the presence of kirromycin or the ribosome, two ligands that are stimulators of the intrinsic GTPase activity of EF-Tu [23]. The absence of any productive interaction with isoGTP strongly suggests the lack of a hydrogen bond interaction between Asn<sup>135</sup> side chain and O(6) of the base, at least of a strength comparable to that between Asp<sup>138</sup> and the exocyclic amino group [7–9]. To test the interaction with other ligands, we also analyzed whether this mutant could sustain the Phe-tRNA<sup>Phe</sup> binding to poly(U)-programmed ribosomes, a sensitive test, since aa-tRNA strongly stabilizes the EF-Tu wt-GTP complex [5]. None of the nucleotides used was capable of inducing any EF-Tu-dependent binding of Phe-tRNA<sup>Phe</sup> to the ribosome, except for GTP in the case of EF-Tu wt, as expected (Fig. 4A). Remarkably, in the presence of kirromycin the enzymatic binding could take place with both EF-Tu species in the absence of any nucleotide (Fig. 4B). It is known that kirromycin induces a GTP-like conformation of EF-Tu, enabling the enzymatic binding of Phe-tRNA<sup>Phe</sup> to the ribosome in the absence of GTP and even in the presence of GDP [24,25]. The observation that kirromycin can induce the same binding with either EF-TuN135D/D138N or EF-Tu wt proves that the lack of interaction with isoGTP and XTP was a consequence of a disordered architecture of the EF-TuN135D/D138N binding pocket and not of a denaturation of the whole molecule.

#### 4. DISCUSSION

The lack of a productive interaction between EF-TuN135D/D138N and the various nucleotides supports a 3D model in which, like the corresponding residue in

p21, Asn<sup>135</sup> is not involved in the recognition of the O(6) group of the base. This residue is crucial for an active conformation of the nucleotide binding pocket, since the replacement of Asn<sup>135</sup> with Asp on EF-TuD138N completely abolishes any response to XTP. Moreover, it is known that substitution of Asn<sup>135</sup>( $\rightarrow$ D) alone induces toxic effects on cell growth [26]. In EF-Tu wt (Fig. 3), Asn<sup>135</sup> connects the loops 19–22 and 173–175 by hydrogen bonds. This interaction takes place between His<sup>22</sup>-OMC (acceptor)⋯Asn<sup>135</sup>-NH<sub>2</sub> (donor) and Asn<sup>135</sup>-O $\beta$ 1 (acceptor)⋯Ala<sup>174</sup>-NH (donor). The substitution with Asp<sup>135</sup>, in which both O $\beta$ 1 and O $\beta$ 2 are acceptors, abolishes the hydrogen bond between His<sup>22</sup> and Asp<sup>135</sup>, indicating that this hydrogen bond is essential for a correct folding of the nucleotide binding pocket.

As derived from the p21 model other residues, such as Ala<sup>174</sup> and perhaps Ser<sup>173</sup>, are likely to be determinants for the O(6) recognition. That O(6) is very important for the EF-Tu recognition of guanine is supported by the observations of Wittinghofer [6] and Eccleston

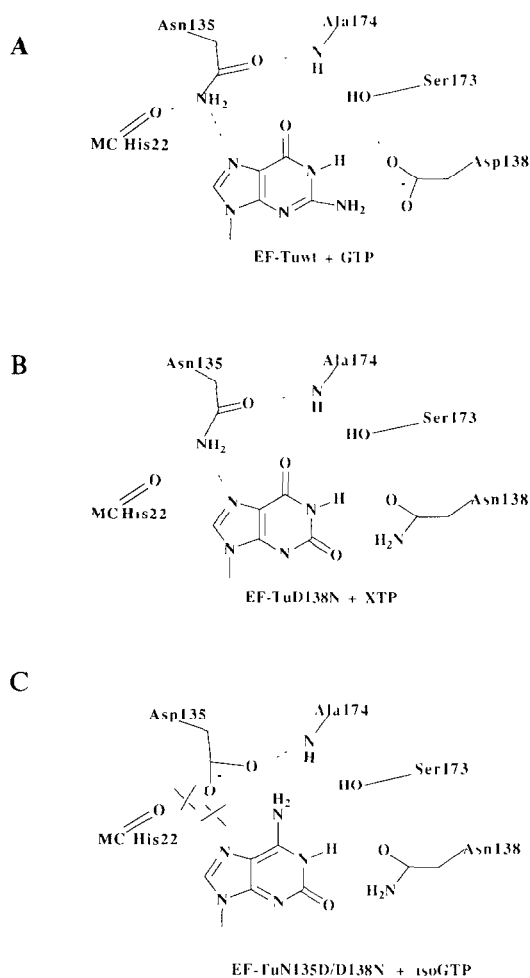


Fig. 3. A diagram representing the hydrogen bond interactions of EF-Tu wt with guanine (A), EF-TuD138N with xanthine (B), and EF-TuN135D/D138N with isoguanine (C), according to the refined model of Kjeldgaard and Nyborg [13].

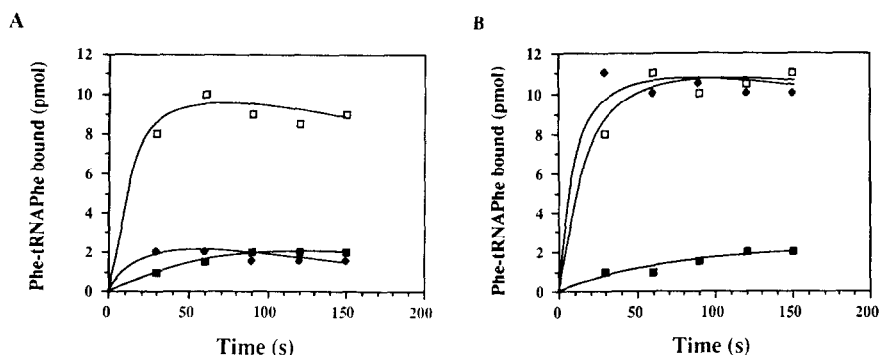


Fig. 4. EF-Tu or EF-TuN135D/D138N mediated binding of Phe-tRNA<sup>Phe</sup> to the ribosome in the presence of GTP (A) or kirromycin (B). The binding was performed by adding to a 30  $\mu$ l reaction mixture (40 pmol *N*-AcetylPhe-tRNA<sup>Phe</sup>-ribosome-Poly(U) complex), 20  $\mu$ l of a solution containing 20 pmol [<sup>3</sup>H]Phe-tRNA<sup>Phe</sup> (1 Ci/mmol), 20 pmol wild-type (□) or mutant (◆) EF-Tu, 20 pmol EF-Ts, and 10  $\mu$ M GTP (A) or 1  $\mu$ M kirromycin (B). The binding experiments were performed at 0°C. At the time intervals indicated samples (9  $\mu$ l) were taken and pipetted onto nitrocellulose filters. EF-Tu-independent (nonenzymatic) binding (■), was carried out as above except that EF-Tu was omitted.

[27] that in p21 replacement of O(6) by a sulphur reduces the affinity towards the nucleotide by two orders of magnitude and its replacement by a hydrogen completely abolishes the binding of the nucleotide. Moreover EF-Tu D138N cannot bind *iso*GTP, whereas EF-Tu D138N is fully active in the presence of XTP. In line with this, though both ITP or XTP can bind to EF-Tu wt with an affinity 100-times lower than GTP [3,6], EF-Tu D138N was unable to perform protein biosynthesis in the presence of ITP (A. Weijland, unpublished results). Therefore for an efficient activity of EF-Tu the interaction with both exocyclic groups is a prerequisite.

Interestingly, despite the anomalous conformation of the nucleotide binding pocket induced by the substitution of Asn<sup>135</sup>, kirromycin is still able to transmit the signal inducing an active GTP-like conformation of EF-Tu, capable of mediating the binding of aa-tRNA to the A site of mRNA-programmed ribosomes. This shows that the mechanism of action of this antibiotic is not conditioned by a correct conformation of the nucleotide binding pocket, but it can propagate via other structures and influence the overall conformation of the EF-Tu molecule.

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