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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^{135}, 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^{135} 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^{135} 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^{138} of EF-Tu [7,8], similarly to the corresponding residue (Asp^{119}) 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^{135} 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^{135} 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^{22}, a residue that is part of the consensus element ^{18}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^{146} (Ala^{174} in EF-Tu), excluding a hydrogen bond with Asn^{116} (Asn^{135} in EF-Tu), a residue that interacts weakly with the N(7) of the base and ties together three fundamental consensus sequence elements (^{10}GXXXXGK; ^{116}NKXD and ^{145}SAK) essential for a correct architecture of the GTP binding pocket. Curi-

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

In the actual state of our 3D knowledge, substitution of both Asn^{135} and Asp^{138} would define the role of Asn^{135} in the nucleotide binding site of EF-Tu and unequivocally elucidate the participation of the side-chain nitrogen of Asn^{135} in the recognition of the base. Indeed, should Asn^{135} 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-TuD138N 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$^+$. The oligodeoxynucleotide GTGTTCCTGGACAAATG was used to substitute Asn^{135} with Asp in EF-TuD138N [12]. The modified *tufA* was overexpressed via pTTQ18 under control of the *tac* promotor in the RecA · *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-$tRNA^{Phe}$ 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 ^1H, ^{13}C, and ^{31}P NMR. The NMR spectra are shown in Fig. 1. [γ-^{32}P] XTP and [γ-^{32}P]*iso*GTP were synthetised by the method of Glynn and Chappell [16], [γ-^{32}P]GTP and [γ-^{32}P]ATP were from Amersham. [^{14}C]Phe-$tRNA^{Phe}$ 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

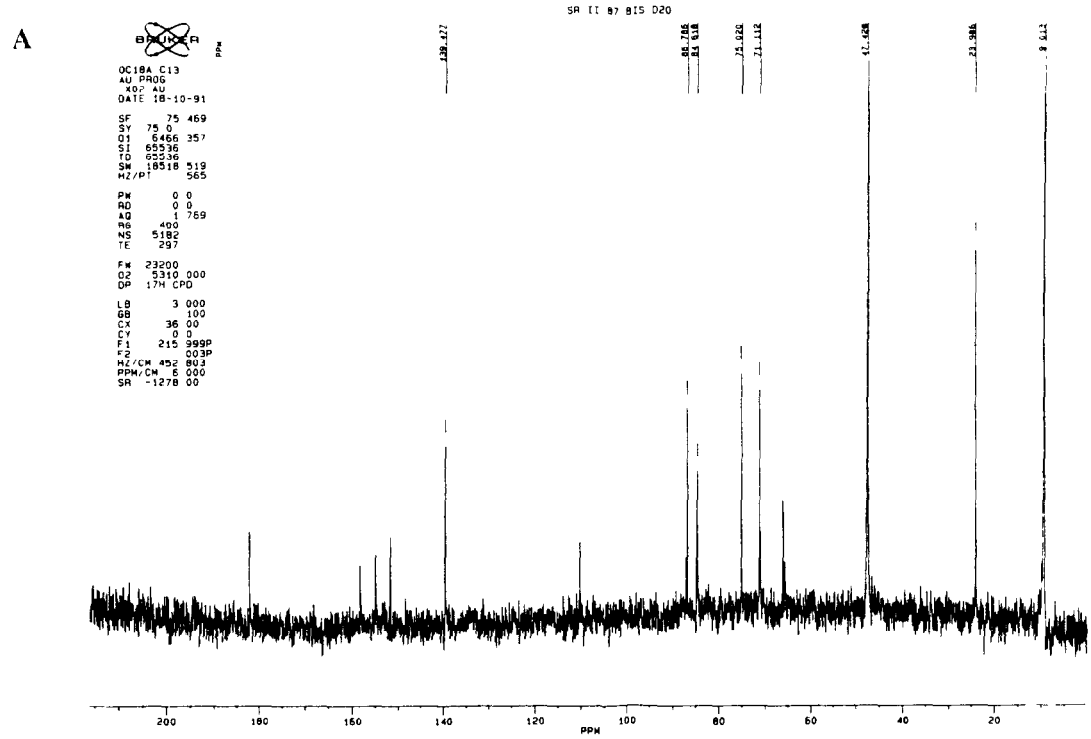

Fig. 1. ^{13}C-, ^{1}H-, ^{31}P-NMR spectra of *iso*GTP. (A) ^{13}C-NMR 75MHz: (D_2O) δ = 65.95(C-5'); 71.08(C-3'); 75.00(C-2'); 84.59(C-4'); 86.75(C-1'); 110.18(C-5); 139.44(C-8); 151.6(C-4); 154.8(C-6); 158.0(C-2).

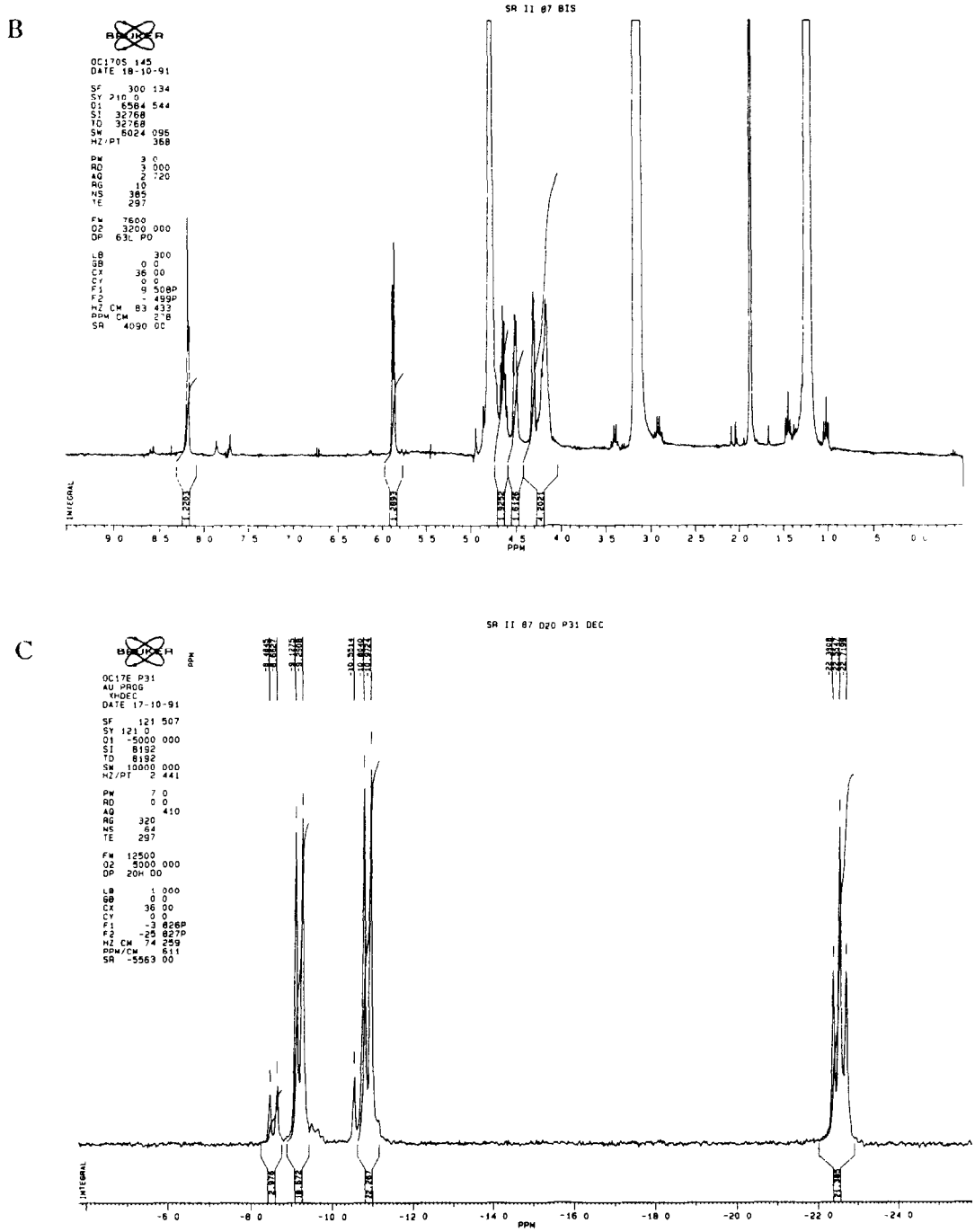

Fig. 1 *(continued)* (B) ^{1}H-NMR 300MHz: (D_2O) δ = 5.87(d, 1H, $J_{1,2}$ = 6.5 Hz, H-1), 8.2(s, 1H, H-8). (C) ^{31}P-NMR 121MHz: (D_2O) d(PO_4H_3 as reference) = −9 2(d, 1P, Pγ, $J_{P\gamma-P\beta}$ = 20.6 Hz); −10,89(d,1P, Pα, $J_{P\alpha-P\beta}$ = 20,6 Hz, $J_{P\alpha-P\gamma}$ = 0 Hz); −22.55(d, 1P, Pβ, $J_{P\beta-P\alpha}$ = $J_{P\beta-P\gamma}$ = 20,6 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 chromatography, 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 °C in 50 mM Tris-HCl buffer, pH 7.6, 70 mM NH_4Cl, 7 mM $MgCl_2$, 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 μM

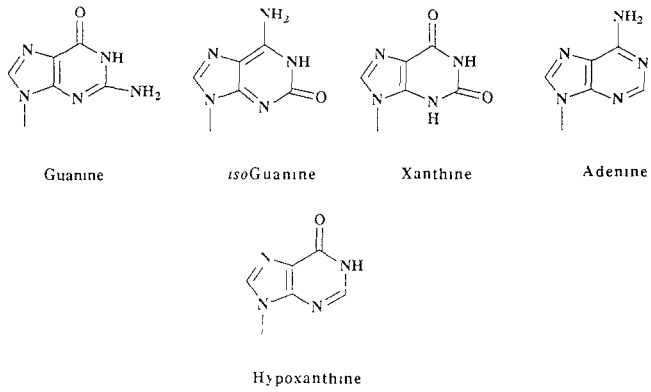

Fig. 2. The structure of the bases of the nucleotides used for this study.

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 [γ-^{32}P]ATP, [γ-^{32}P]XTP, [γ-^{32}P]GTP or [γ-^{32}P]*iso*GTP, 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 *iso*GTP strongly suggests the lack of a hydrogen bond interaction between Asn^{135} side chain and O(6) of the base, at least of a strength comparable to that between Asp^{138} 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^{Phe} 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^{Phe} 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^{Phe} 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 *iso*GTP 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/N138N and the various nucleotides supports a 3D model in which, like the corresponding residue in p21, Asn^{135} 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^{135} with Asp on EF-TuD138N completely abolishes any response to XTP. Moreover, it is known that substitution of Asn^{135}(→D) alone induces toxic effects on cell growth [26]. In EF-Tu wt (Fig. 3), Asn^{135} connects the loops 19–22 and 173–175 by hydrogen bonds. This interaction takes place between His^{22}-OMC (acceptor)···Asn^{135}-NH$_2$ (donor) and Asn^{135}-Oβ1 (acceptor)···Ala^{174}-NH (donor). The substitution with Asp^{135}, in which both Oβ1 and Oβ2 are acceptors, abolishes the hydrogen bond between His^{22} and Asp^{135}, 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^{174} and perhaps Ser^{173}, 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

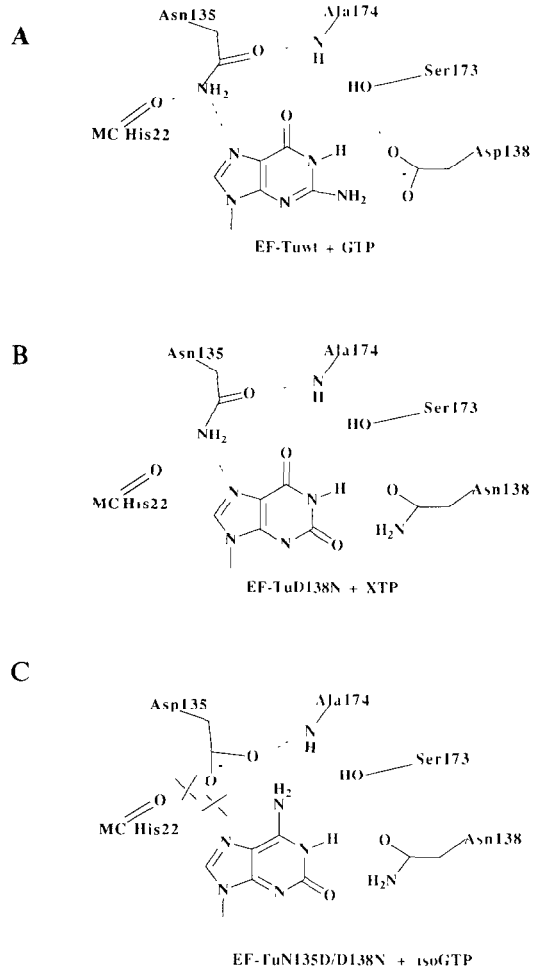

Fig. 3. A diagram representing the hydrogen bond interactions of EF-Tu wt with guanine (A), EF-TuD138N with xanthine (B), and EF-TuN135D/D138N with *iso*guanine (C), according the refined model of Kjeldgaard and Nyborg [13].

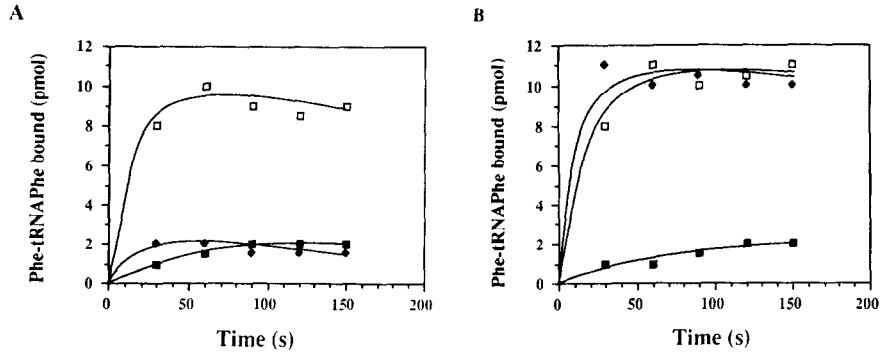

Fig. 4. EF-Tu or EF-TuN135D/D138N mediated binding of Phe-tRNA^{Phe} to the ribosome in the presence of GTP (A) or kirromycin (B). The binding was performed by adding to a 30 μl reaction mixture (40 pmol *N*-AcetylPhe-tRNAPhe·ribosome·Poly(U) complex), 20μlof a solution containing 20 pmol [^{3}H]Phe-tRNA^{Phe} (1 Ci/mmol), 20 pmol wild-type (□) or mutant (◆) EF-Tu , 20 pmol EF-Ts, and 10 μM GTP (A) or 1 μM kirromycin (B). The binding experiments were performed at 0°C. At the time intervals indicated samples (9 μl) 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-TuD138N 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^{135}, 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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