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Mutations to kirromycin resistance occur in the interface of domains I and III of EF-Tu·GTP

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Abstract The antibiotic kirromycin inhibits protein synthesis by binding to EF-Tu and preventing its release from the ribosome after GTP hydrolysis. We have isolated and sequenced a collection of kirromycin resistant *tuf* mutations and identified thirteen single amino acid substitutions at seven different sites in EF-Tu. These have been mapped onto the 3D structures of EF-Tu·GTP and EF-Tu·GDP. In the active GTP form of EF-Tu the mutations cluster on each side of the interface between domains I and III. We propose that this domain interface is the binding site for kirromycin.

Key words: EF-Tu; Kirromycin; Protein structure; *tuf* mutation; *Salmonella typhimurium; Escherichia coli*

1. Introduction

EF-Tu is an essential component of bacterial protein biosynthesis and forms a ternary complex with GTP and aminoacyl-tRNA [1]. This complex interacts with the elongating ribosome, placing aminoacyl-tRNA in the A-site. After codon–anticodon interaction, GTP on EF-Tu is hydrolysed, causing the dissociation of EF-Tu·GDP from aa-tRNA and from the ribosome. EF-Tu·GDP is subsequently recycled to an active form via a nucleotide exchange reaction promoted by EF-Ts, after which it can again form a ternary complex with aa-tRNA [2]. High resolution crystallographic structures of EF-Tu in its GDP and GTP conformations have recently been solved [3–5]. Kirromycin is an antibiotic of MW 797 which affects protein synthesis by binding to EF-Tu and inhibiting the release of EF-Tu·GDP from the ribosome, thus preventing elongation from progressing [6]. Kirromycin is known from in vitro studies to interact with both the EF-Tu·GDP and EF-Tu·GTP forms of the factor.

Several mutants resistant to kirromycin have previously been isolated in *Salmonella typhimurium* and *Escherichia coli*. Most mutations causing kirromycin resistance map in the genes for EF-Tu, *tufA* and *tufB* [7,8], and sequence alterations causing five changes at four positions have been identified and reported [9–12]. Mutations causing kirromycin resistance have also been identified in *rpsL*, as error restrictive mutants of ribosomal protein S12 [13]. In an attempt to define the kirromycin binding region of EF-Tu we have isolated a large number of spontaneous kirromycin resistant mutants, identified the sequence alterations, and mapped these residues, and those of the previously identified mutants, onto the 3D structures of EF-Tu [3,4]. The kirromycin resistant mutations are caused individually by thirteen amino acid substitutions at seven different residues, and one amino acid deletion at another site. The mutations map in the structural domains I and III. All of the amino acid substitutions are in the domain I–III interface when EF-Tu is in the GTP conformation, but are dispersed when EF-Tu is in its GDP conformation. The deletion maps in the loop connecting domains II and III.

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2. Materials and methods

2.1. Selection and identification of mutations

Kirromycin resistant mutants were selected spontaneously on LC media [7] containing 2 mM EDTA (pH 8.0) and 100 μg/ml kirromycin at 30°C with *S. typhimurium*. The strains used for most of the selections carried only one active *tuf* gene (TH381, *trpE*91 *tufA*300::MudJ; and TH701, *trpE*91 *tufB*441::MudJ) but one mutation was selected in TH488 (*trpE*91 *hisG*3720 *tufA*8). Mutations causing resistance to kirromycin were mapped by transduction and the relevant *tuf* gene sequenced after asymmetric PCR amplification.

2.2. PCR and sequencing

The PCR oligos and amplification protocols were as described previously [11] with the following modifications. DNA for PCR amplification was prepared by suspending a bacterial colony in 50 μl distilled water in a microfuge tube, incubating for 30 min at 37°C (the cells swell), adding 5 μl of this solution to a standard PCR buffer and primer mixture and subjecting this to PCR (the swollen cells burst and release their DNA). The DNA oligos used for *tufA* amplification were Sal3′ (+42 to +20 nt downstream of *tufA*, 5′CCGAAGCGCCCTCTTCAATTCAAA3′); 5′ efg-end (the last eight codons in *fusA* plus the termination triplet [14], 5′GCCGTAATTGAAGCCCGTGGTAAATAA3′); and TA5′ (−54 to −30 upstream of *tufA* [14], 5′CCAAAATCCCGTGCTCTCTCCTGAA3′). The Sal3′ sequence information was obtained by inverse PCR and sequencing of the region immediately downstream of *tufA* in *S. typhimurium*. The DNA oligos used for *tufB* ampification, and for sequencing both *tuf* genes have been described previously [11]. Asymmetric PCR products were purified using a GeneClean kit and sequenced by the dideoxy method using T7 DNA polymerase (Pharmacia) and [α-^{35}S]dATP.

3. Results and discussion

3.1. Mutant isolation and identification

We have isolated kirromycin resistant mutations in the *tufA* and *tufB* genes of *S. typhimurium*. By DNA sequencing we have identified twelve different kirromycin resistant mutations. We have also sequenced a previously characterized kirromycin resistant mutation in *tufA* of *E. coli*, *tufAa* [15], and identified it as a novel mutation. These thirteen mutations map in eight different codons, in seven of which a single amino acid substitution results, while in one case a single amino acid is deleted. Four *tuf* mutations causing kirromycin resistance have been identified in *E. coli* [9,12]. We have isolated three of these mutations also in *S. typhimurium*, while the fourth one affects residue 124 where we have isolated two different mutations. All

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fourteen mutations are listed in Table 1, where we give the amino acid residue numbers in *E. coli* and *S. typhimurium* (identical residues) and in *Thermus aquaticus* (contains three small insertions relative to *E. coli*) to facilitate comparison of the GDP and GTP structural forms of EF-Tu [4]. Throughout this report we use the *E. coli* numbering in the text and in the figures for simplicity. Residues 120, 124 and 160 are in domain I of EF-Tu, while residues 316, 329, 375 and 378 are in domain III. The amino acid deleted (residue 298) is in the loop connecting domains II and III. Each of these mutant forms of EF-Tu can individually support bacterial viability. No similar viable kirromycin resistant mutations have been isolated in domain II. Those mutations giving rise to kirromycin resistance that have been isolated in domain II are all individually inviable and essentially serve to expose the phenotype of a viable kirromycin resistance mutation on the other *tuf* gene [11,16], although they may be at least partially active in the functions of EF-Tu [16–18]. Kirromycin has been crosslinked by borohydride reduction to residue Lys^{357} in domain III of *E. coli* EF-Tu [19].

3.2. Location of kirromycin resistance mutations on the 3D structures of EF-Tu

We have mapped each of the residues where a substitution causes kirromycin resistance (and the crosslinked residue) onto the EF-Tu·GDP structure from *E. coli* [3] and onto the EF-Tu·GTP structure from *Thermus aquaticus* [4]. The results are shown in Fig. 1A and B. In this figure, the orientation of the guanine nucleotide binding domain I is kept constant, while the relative orientation of domains II and III changes considerably depending on the nature of the bound nucleotide, as discussed by Kjeldgaard et al. [4]. In the GTP form (Fig. 1B) the seven residues where substitutions cause resistance to kirromycin cluster in the interface between domains I and III. In contrast, in the GDP form (Fig. 1A) the seven residues are widely dispersed, the three domain I mutations being still in the interface, while the domain III mutations are turned away because of the altered orientation of the domains. We suggest that the most parsimonious interpretation

Table 1
Mutations in EF-Tu causing resistance to kirromycin

Mutant position		aa change	nt change
E.c., S.t.	T.a.		
120	121	L, Leu→Gln	CTG→CAG
124	125	Q, Gln→Arg	CAG→CGG
		Q, Gln→Glu	CAG→GAG
		Q, Gln→Lys*	CAG→AAG
160	161	Y, Tyr→Asn	TAC→AAC
		Y, Tyr→Asp	TAC→GAC
		Y, Tyr→Cys	TAC→TGC
298	310	I, Δ Ile	Δ ATT
316	328	G, Gly→Asp	CGC→GAC
329	341	Q, Gln→His	CAG→CAC
375	387	A, Ala→Thr	GCA→ACA
		A, Ala→Ser	GCA→TCA
		A, Ala→Val	GCA→GTA
378	390	E, Glu→Lys*	GAA→AAA

E.c., S.t. and T.a. refer to amino acid residue numbers in *Escheria coli*, *Salmonella typhimurium* and *Thermus aquaticus* respectively. *These mutations have so far only been isolated in *E. coli*. The mutation Gln124Lys was isolated and identified by Zeef and Bosch [12], the mutation tufAa was isolated by Tapio and Isaksson [15] and the sequence change Glu378Lys identified by us.

of these locations on the structure is that kirromycin binds in vivo to the interface of domains I and III of EF-Tu·GTP. According to this interpretation the mutations in the domain interface would cause resistance by interfering with the binding of kirromycin. The amino acid deletion in the loop linking domains II and III may cause resistance indirectly, by modifying the conformation of the proposed binding site in the interface between domain I and III. The crosslinking of kirromycin to Lys^{357} in the *E. coli* EF-Tu is interesting. This lysine residue (Thr in *Thermus aquaticus*) is in the domain I–III interface in the EF-Tu·GDP conformation, but not in the EF-Tu·GTP conformation (Fig. 1A and B). This is compatible with kirromycin occupying the domain I–III interface in the EF-Tu·GDP form also, bringing Lys^{357} into the immediate neighbourhood of kirromycin. Kirromycin binds to both forms of EF-Tu in vitro [6], causing a hybrid conformation with characteristics of both GDP and GTP conformations [20,21]. Our data would suggest that the interaction of kirromycin with EF-Tu need not neccessarily depend on specific interactions with residues on domain III as these would differ in the two forms of EF-Tu. This suggests the possibility that some of the domain I residues (124, 120 and 160), or residues in their immediate structural neighbourhood, may be directly involved in binding kirromycin to EF-Tu. Note that the size of the kirromycin molecule (MW 797, Fig. 2) is such that it could, in elongated form, easily cover the length of the interface from Tyr^{160} to Gln^{124}. Studies of the complex of kirromycin and EF-Tu suggest that two regions of the antibiotic, the chromophore N(1)–C(13) and the alkene region C(18)–C(24), are involved in binding [22–24].

3.3. Local structural consequences of the kirromycin resistance mutations in EF-Tu·GTP

The three amino acid residues giving rise to kirromycin resistance mutations in domain I, Tyr^{160}, Leu^{120} and Gln^{124}, are immediate neighbours in the 3D structure (Figs. 1A, 1B, 3A) and have side chains pointing in the same direction towards domain III. Tyr^{160} has van der Waals interactions with Leu^{120} and also hydrogen bonds with Arg^{116} and with Glu^{315} in domain III (Fig. 3A and B). The mutations of Tyr^{160} to Asn, Asp and Cys (all of which have smaller side chains than Tyr) are each likely to result in the loss of these hydrogen bonds. Leu^{120} (Fig. 3A) makes van der Waals interactions with Tyr^{160} and has a hydrophobic interaction with Leu^{311} in domain III. The mutation Leu^{120}→Gln can be expected to replace this hydrophobic interaction with a hydrogen bond interaction to another polar side chain in the interface. Gln^{124} (Fig. 3A,C,D) hydrogen bonds to the main chain of Ala^{375} in domain III (note that Ala^{375} is one of the mutant sites on domain III). Of the three mutations isolated at this position, Gln^{124} to Arg, Glu and Lys, the Arg and Lys mutants may have polar interactions with other groups in the interface.

There are four residues in domain III where substitutions were isolated, Gly^{316}, Gln^{329}, Ala^{375} and Glu^{378}. The mutation Gly^{316}→Asp is likely to create a hydrogen bond to Tyr^{160} and possibly also a salt bridge to Arg^{117}, both in domain I. The local consequences of the mutation Gln^{329}→His are more difficult to predict. This residue is probably hydrogen bonded to Asp^{336} and Thr^{338} and these interactions may be maintained in the His mutant. Ala^{375} is hydrogen bonded through its main chain to Gln^{124} in domain I. Two of the mutations at this position, Ala^{375} to Thr and Ser, may form hydrogen bonds to the side chain of

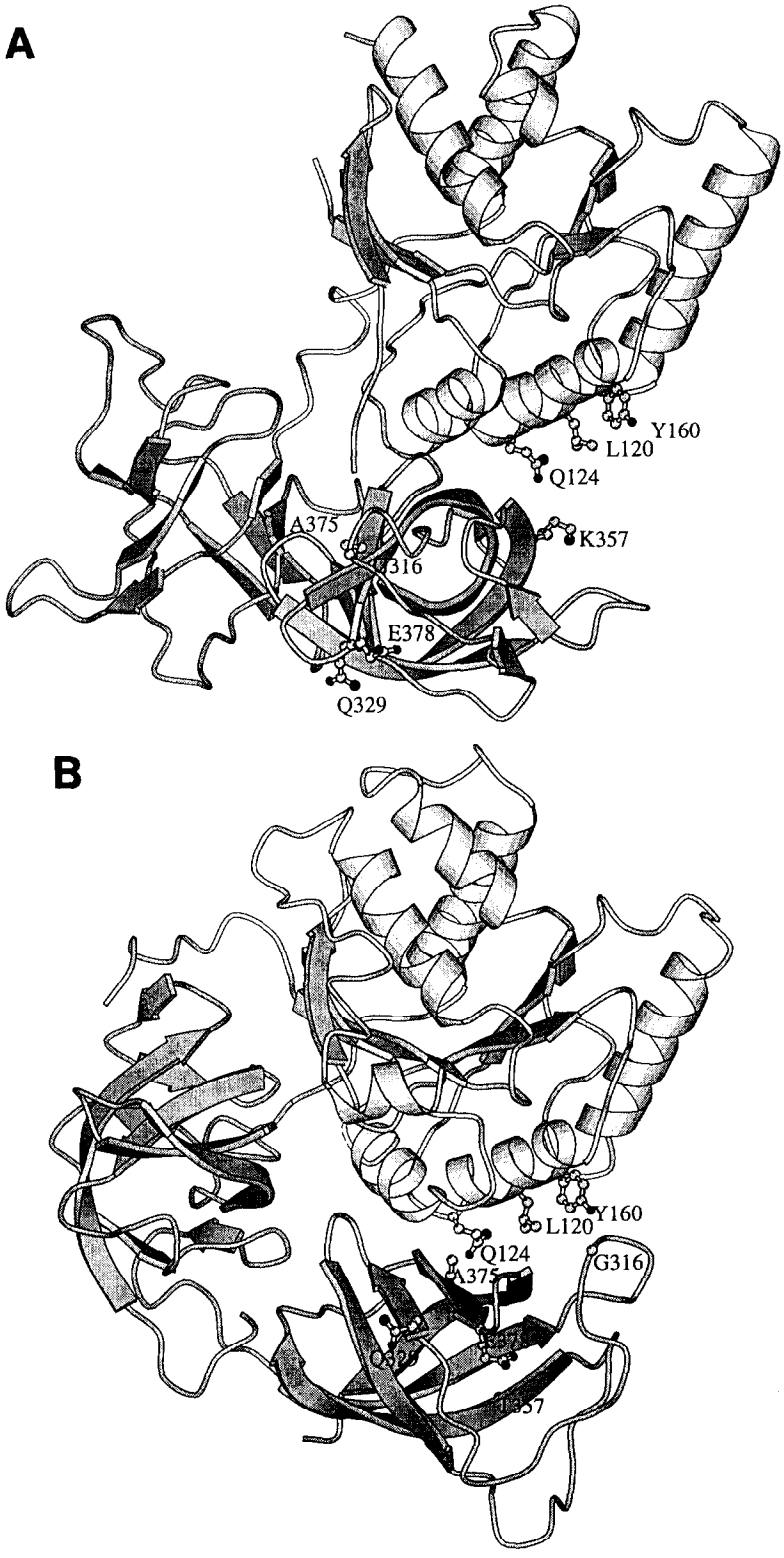

Fig. 1. (A) Structure of EF-Tu·GDP from *E. coli* [3] and (B) EF-Tu in its GTP conformation from *Thermus aquaticus* [4] showing the positions and side chains of amino acids where mutations causing resistance to kirromycin have been isolated, and the site, 357, of borohydride crosslinking to kirromycin [19]. Note that all residues are numbered according to *E. coli* EF-Tu.

Gln^{124}. The third mutant, $Ala^{375} \rightarrow Val$, may make hydrophobic interactions with Leu^{121}. Glu^{378} makes a salt bridge to Arg^{318} and a hydrogen bond with Tyr^{326}. The mutation $Glu^{378} \rightarrow Lys$ will cause the loss of this salt bridge and of the hydrogen bonding interaction. Thus, the overall conclusion is that many of the amino acid substitutions causing kirromycin resistance

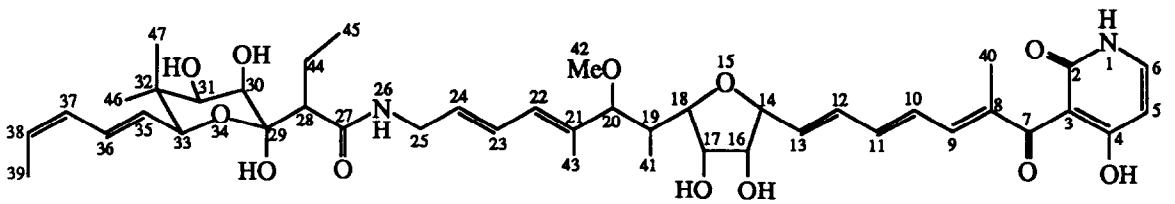

Fig. 2. Chemical structure of kirromycin. MW 797.

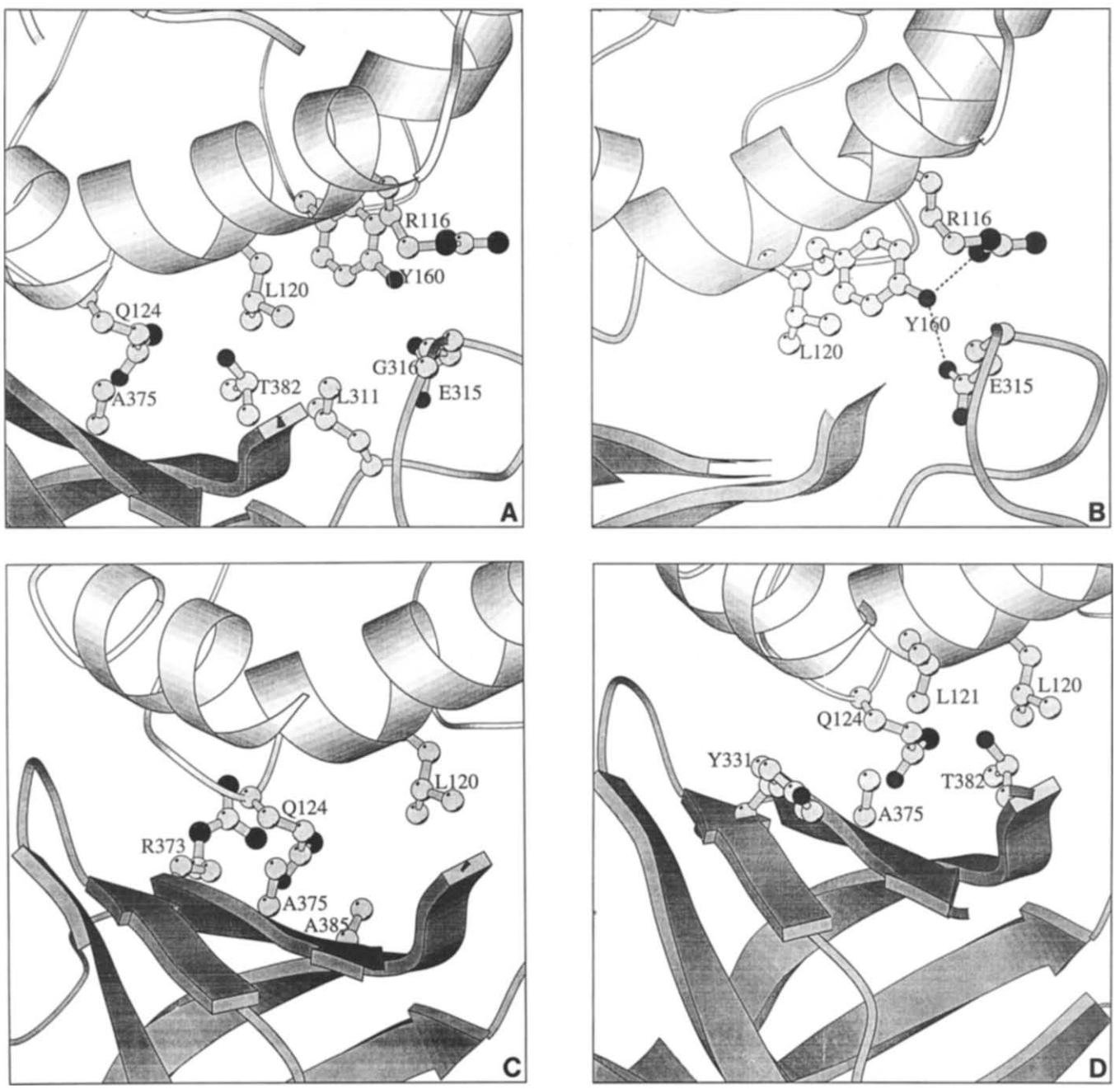

Fig. 3. Residues in the interface of domains I and III of EF-Tu in the GTP conformation. Note that all residues are numbered according to *E. coli* EF-Tu. (A) Overview of residues Y160, L120 and Q124, (B) Y160 hydrogen bonding, (C) Q124 neighbourhood, (D) A375 neighbourhood.

may affect the strength of the interactions between domains I and III of EF-Tu.

3.4. Secondary phenotypes

Most of the kirromycin resistant substitution mutations also have secondary phenotypes, which probably reflects the extreme conservation and functional complexity of EF-Tu and its interactions. Increased translational errors, defective interactions with aminoacyl-tRNA or with the ribosome are some of the phenotypes associated with some of the previously described substitutions at positions 375 and 378 [25–27]. Note that the affinity of EF-Tu for aminoacyl-tRNA is also drastically reduced by kirromycin binding [21]. We are currently investigating the secondary phenotypes associated with several of the new kirromycin resistant mutants described in this report.

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