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Kirromycin, an Inhibitor of Protein Biosynthesis that Acts on Elongation Factor Tu

(EF-Tu GTPase/peptide bond formation/aminoacyl-tRNA binding/ribosomal complexes)

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ABSTRACT Kirromycin, a new inhibitor of protein synthesis, is shown to interfere with the peptide transfer reaction by acting on elongation factor Tu (EF-Tu). All the reactions associated with this elongation factor are affected. Formation of the EF-Tu·GTP complex is strongly stimulated. Peptide bond formation is prevented only when Phe-tRNA^{Phe} is bound enzymatically to ribosomes, presumably because GTP hydrolysis associated with enzymatic binding of Phe-tRNA^{Phe} is not followed by release of EF-Tu·GDP from the ribosome. This antibiotic also enables EF-Tu to catalyze the binding of Phe-tRNA^{Phe} to the poly(U)·ribosome complex even in the absence of GTP. EF-Tu activity in the GTPase reaction is dramatically affected by kirromycin: GTP hydrolysis, which normally requires ribosomes and aminoacyl-tRNA, takes place with the elongation factor alone. This GTPase shows the same K_m for GTP as the one dependent on Phe-tRNA^{Phe} and ribosomes in the absence of the antibiotic. Ribosomes and Phe-tRNA^{Phe}, but not tRNA^{Phe} or Ac-Phe-tRNA^{Phe}, stimulate the kirromycin-induced EF-Tu GTPase. These results indicate that the catalytic center of EF-Tu GTPase that is dependent upon aminoacyl-tRNA and ribosomes is primarily located on the elongation factor. In conclusion, kirromycin can substitute for GTP, aminoacyl-tRNA, or ribosomes in various reactions involving EF-Tu, apparently by affecting the allosteric controls between the sites on the EF-Tu molecule interacting with these components.

In 1972 the new antibiotics kirromycin (1), mocimycin (2, 3), and X-5108 (4, 5) were isolated from cultures of different *Streptomyces*. Kirromycin and mocimycin turned out to have the same chemical structure, while the antibiotic X-5108 was identified as the *N*-methylated form (6, 7). Their structure did not seem to be related to any of the known classes of antibiotics. Kirromycin was found to inhibit the growth of some species of bacteria and poly(U)-directed poly(phenylalanine) synthesis in the *in vitro* system of *Escherichia coli* (8). In an attempt to localize the site of this inhibition, we have examined the action of this antibiotic on the partial steps of polypeptide chain elongation. Our results show that kirromycin inhibits the peptidyl transfer reaction by acting on the elongation factor Tu (EF-Tu) and is thus the first antibiotic found to have EF-Tu as its target. Kirromycin has proved to be a very useful tool for investigating EF-Tu-dependent reactions. Its ability to induce GTPase activity with EF-Tu alone in the absence of ribosomes indicates that the catalytic center for EF-Tu-dependent ribosomal GTP hydrolysis is primarily situated on the elongation factor.

Abbreviations: EF-Tu, elongation factor Tu; EF-Ts, elongation factor Ts; EF-T, elongation factor T, the complex formed by EF-Tu and EF-Ts; EF-G, elongation factor G.

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MATERIALS AND METHODS

EF-Tu and elongation factor Ts (EF-Ts) were isolated from purified elongation factor T (EF-T is the complex formed by EF-Tu and EF-Ts) by chromatography on DEAE–Sephadex A-50 in the presence of 0.05 mM GDP (9). Homogeneous EF-T and elongation factor G (EF-G) were isolated essentially as described (10). In most experiments we have used EF-T, since association with EF-Ts eliminates both the need of GDP for stability of EF-Tu and the difficulties connected with the complete removal of this nucleotide before assay. In the *Results* section, we have preferred in a few instances to refer to EF-T when this factor was used even though the effects observed were related to the EF-Tu component. One microgram of EF-G, EF-T, EF-Tu, and EF-Ts was taken to correspond to 12, 15, 24, and 35 pmol, respectively (11, 12). NH_4Cl-washed ribosomes were prepared as reported (10). One A_{260} unit of ribosomes is equivalent to 25 pmol (13). Highly purified phenylalanyl-tRNA synthetase, tRNA^{Phe} (50–60% pure), Phe-tRNA^{Phe}, and Ac-Phe-tRNA^{Phe} were prepared as reported (14). Protein determinations were carried out according to Lowry *et al.* (15) by use of crystalline bovine serum albumin as the standard. GTP, GDP, ATP, and poly(U) were purchased from Boehringer (Mannheim); [^{3}H]GTP and [^{14}C]phenylalanine were from The Radiochemical Centre (Amersham); and [γ-^{32}P]GTP was prepared as reported (16). Purity of the labeled nucleotides was checked by thin layer chromatography (17). Kirromycin (molecular weight 794, ref. 6) was isolated as described (1). On storage in ethanolic solution at −25° in the dark, it was stable for at least several months. Its concentration was determined by weight and spectrophotometrically ($E^{1\%}_{325}$ = 355 in 0.1 M NaOH–ethanol 1:1, ref. 2).

All reactions were performed in standard buffer [60 mM Tris·HCl (pH 7.8)–30 mM KCl–30 mM NH_4Cl–10 mM $MgCl_2$–2 mM dithiothreitol] with 0.3–1% glycerol (carried over with the factors and ribosomes) and 1–2% ethanol (from kirromycin). The same amount of ethanol was added to the controls. Incorporation of [^{14}C]phenylalanine into hot trichloroacetic acid-insoluble material and GTP hydrolysis were measured as described (14). Binding of [^{3}H]GTP or [γ-^{32}P]GTP to EF-Tu and of [^{14}C]Phe-tRNA^{Phe} to ribosomes were determined by filtration through nitrocellulose filters (Millipore HAWP 0.45 μm). Filters were washed with 4 ml of standard buffer.

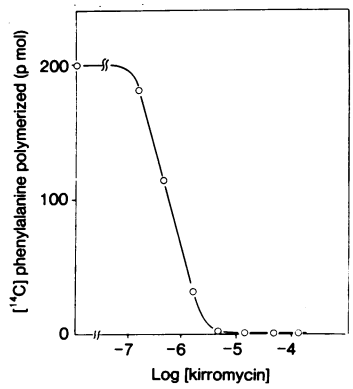

FIG. 1. Inhibition of poly(U)-directed poly(phenylalanine) synthesis as a function of kirromycin concentration. Reaction mixture A contained 200 pmol of ribosomes and 20 μg of poly(U) in 0.5 ml of standard buffer. Reaction mixture B contained 1.2 nmol of $tRNA^{Phe}$, 15 μg of phenylalanyl-tRNA synthetase, 450 pmol of EF-T, 75 pmol of EF-G, 7 nmol of [^{14}C]phenylalanine (50 Ci/mol), 250 nmol of ATP, and 250 nmol of GTP in 250 μl of standard buffer. Reaction mixtures A and B were incubated for 5 min at 30° to allow formation of poly(U)·ribosome complex and [^{14}C]-Phe-$tRNA^{Phe}$, respectively, and then chilled to 0°. Five microliters of a 20% ethanol solution with or without kirromycin and 20 μl of mixture B were added to 50 μl of mixture A. The samples were incubated for 5 min at 30° and assayed for incorporation of [^{14}C]phenylalanine into hot trichloroacetic acid-insoluble material.

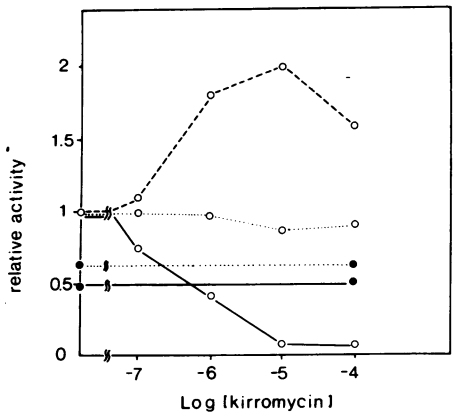

FIG. 2. Effect of kirromycin on Phe-$tRNA^{Phe}$ binding to ribosomes, on EF-T GTPase, and on peptide transfer reaction. Ribosomes (29 pmol) with poly(phenylalanyl)-$tRNA^{Phe}$ in the donor site, 60 pmol of [^{14}C]Phe-$tRNA^{Phe}$ (513 Ci/mol), and, where indicated, 160 pmol of [γ-^{32}P]GTP (1800 Ci/mol), 28 pmol of EF-T, and kirromycin were incubated for 5 min at 30° in 200 μl of standard buffer. Binding of [^{14}C]Phe-$tRNA^{Phe}$ to ribosomes (dotted curves), GTP hydrolysis (dashed curves), and COOH-terminal incorporation of [^{14}C]phenylalanine into poly(phenylalanine) (solid curves) were determined with 60-μl aliquots of the same reaction mixture. Activities with (○) and without (●) EF-T plus GTP. The values obtained in the presence of EF-T and GTP without kirromycin corresponded, per 60-μl aliquot, to 6.1 pmol of [^{14}C]Phe-$tRNA^{Phe}$ bound to ribosomes, 8.1 pmol of GTP hydrolyzed, and 4.2 pmol of [^{14}C]phenylalanine incorporated into polypeptides.

Ribosomes carrying poly(phenylalanyl)-$tRNA^{Phe}$ in the donor site were prepared by poly(U)-directed phenylalanine incorporation in the presence of a rate-limiting amount of Phe-$tRNA^{Phe}$. Under this condition, all poly(phenylalanine) was bound to the donor site since EF-G plus GTP did not stimulate the puromycin reaction. The reaction mixture contained, in 1 ml of standard buffer: 2 nmol of ribosomes, 130 μg of poly(U), 10 nmol of $tRNA^{Phe}$, 20 μg of phenylalanyl-tRNA synthetase, 2.4 nmol of EF-T, 0.6 nmol of EF-G, 1 μmol of ATP, 0.5 μmol of GTP, and 15 nmol of phenylalanine. After an incubation of 20 min at 30°, the reaction mixture was chilled and passed through a Sepharose 4B (Pharmacia, Uppsala) column (1.5 × 30 cm) equilibrated with standard buffer. The excluded peak contained the polysomes carrying most of the poly(phenylalanyl)-$tRNA^{Phe}$.

For further details of the experimental conditions, see the legends of the figures and tables.

RESULTS

Localization of Kirromycin Action. Kirromycin strongly inhibits poly(U)-directed poly(phenylalanine) synthesis in the *E. coli* system, a concentration of 5×10^{-7} M being sufficient for a 50% inhibition (Fig. 1).

To localize the action of this antibiotic, we examined its effect on the single steps of polypeptide chain elongation. In the series of experiments illustrated in Fig. 2, ribosomes carrying unlabeled poly(phenylalanyl)-$tRNA^{Phe}$ in the donor site were incubated with [^{14}C]Phe-$tRNA^{Phe}$ in the presence or absence of EF-T and [γ-^{32}P]GTP. EF-T-directed binding of [^{14}C]Phe-$tRNA^{Phe}$ to the ribosomal acceptor site was hardly affected by kirromycin, while the EF-T-dependent GTPase was stimulated up to twofold and the peptidyl transfer reaction was strongly inhibited. In the absence of EF-T, both the [^{14}C]Phe-$tRNA^{Phe}$ binding and peptidyl-transfer reaction were not affected by the antibiotic. These results show that kirromycin allows enzymatic and nonenzymatic binding of aminoacyl-tRNA to ribosomes but, in the presence of EF-T, inhibits the functional interaction of the C-C-A-aminoacyl end of the tRNA with the acceptor region of the peptidyltransferase center.

In experiments not shown, the ability of peptidyl-tRNA or Ac-Phe-$tRNA^{Phe}$ to react with puromycin (peptidyltransferase activity), as well as the ability of EF-G plus GTP to stimulate the puromycin reaction (translocation activity), were found to be unaffected by kirromycin. EF-G-dependent ribosomal GTPase activity was only inhibited with high concentrations of the antibiotic: in the presence of 10^{-4} M kirromycin, a 15–20% inhibition was observed.

Action of Kirromycin on EF-Tu-Dependent Reactions. Since the action of kirromycin in polypeptide synthesis appeared to be mediate by EF-T, we examined in detail its effect on the EF-T-dependent reactions. As shown in Fig. 3, the antibiotic stimulated the binding of [^{3}H]GTP to EF-T more than threefold, while the relative amount of the resulting EF-Tu·GTP complex released from nitrocellulose filters by Phe-$tRNA^{Phe}$ was essentially not affected. Similar results were obtained when [γ-^{32}P]GTP was used.

EF-T-dependent GTPase activity normally requires ribosomes and aminoacyl-tRNA, and is stimulated by mRNA (16, 18, 19). However, as Table 1 illustrates, kirromycin was able to induce GTP hydrolysis with EF-T alone. This GTP hydrolysis was stimulated by Phe-$tRNA^{Phe}$ but not by $tRNA^{Phe}$ or Ac–Phe–$tRNA^{Phe}$. Poly(U) alone or in combination with Phe-$tRNA^{Phe}$ had no effect. Addition of ribosomes strongly enhanced the kirromycin-induced EF-T GTPase.

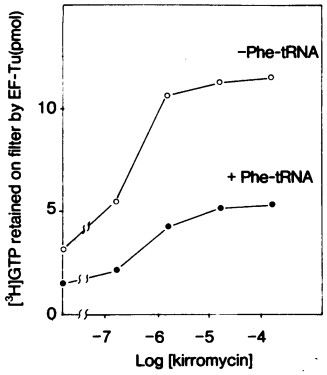

FIG. 3. Effect of kirromycin on the formation of EF-Tu·GTP and on its interaction with Phe-tRNA^{Phe}. Five hundred pmol of EF-T and 1.3 nmol of [^{3}H]GTP (400 Ci/mol) were incubated for 5 min at 30° in 550 μl of standard buffer and chilled to 0°. To 50-μl aliquots of the reaction mixture, 10 μl of standard buffer containing, where indicated, 35 pmol of Phe-tRNA^{Phe} and kirromycin were added. After 5 min of incubation at 0°, 50 μl were withdrawn and analyzed by filtration through nitrocellulose filters. [^{3}H]GTP retained on filter with (●) and without (○) Phe-tRNA^{Phe}.

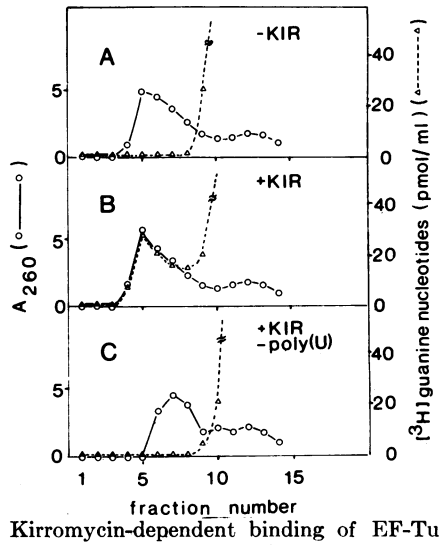

FIG. 4. Kirromycin-dependent binding of EF-Tu to ribosomes as measured by gel filtration. Ribosomes (98 pmol), 150 pmol of EF-T, 150 pmol of Phe-tRNA^{Phe}, 140 pmol of [^{3}H]GTP (400 Ci/mol), and, where indicated, 10 μg of poly(U) and 1 nmol of kirromycin (KIR) were incubated for 5 min at 30° in 150 μl of standard buffer. The reaction mixtures were chilled to 0° and analyzed by gel filtration on Sepharose 4B columns (0.7 × 8 cm) equilibrated with standard buffer. Fractions of 150 μl were collected. Aliquots (100 μl each) were dissolved in 5 ml of Aquasol (New England Nuclear, Boston) and analyzed for radioactivity (△). Absorbance at 260 nm was determined on 20-μl aliquots after dilution (○).

Phe-tRNA^{Phe} decreased the amount of ribosomes needed for maximal stimulation, but the additional presence of poly-(U) reduced this effect. With the higher concentration of ribosomes, the combination of poly(U) plus Phe-tRNA^{Phe} became inhibitory.

The K_m for GTP of the kirromycin-induced GTPase reaction with EF-T alone was found both in the presence and absence of Phe-tRNA^{Phe} to be 2×10^{-7}M, which is identical to the K_m of ribosome- and Phe-tRNA^{Phe}-dependent EF-T GTPase in the absence of the antibiotic (18).

Since EF-Tu is the component of EF-T responsible for GTP binding and hydrolysis (20–23), all the results presented up to now indicate that EF-Tu is the component of EF-T affected by kirromycin. This is confirmed by the experiments presented in Table 2. EF-Ts in the presence of kirromycin appeared to only slightly stimulate the EF-Tu-dependent GTPase reaction.

The experiments described in Fig. 4 suggest that in the presence of poly(U) and Phe-tRNA^{Phe}, kirromycin prevents the release of EF-Tu·GDP from ribosomes after GTP hydrolysis. In fact, the labeled nucleotide from a reaction mixture containing EF-T, ribosomes, poly(U), Phe-tRNA^{Phe}, [^{3}H]GTP, and kirromycin chromatographed with ribosomes on Sepharose 4B (Fig. 4B). No radioactivity was found associated with ribosomes when kirromycin or poly(U) were omitted from the system (Fig. 4A and C). The nucleotide bound to ribosomes was identified as GDP, since when [^{3}H]-GTP was replaced with [γ-^{32}P]GTP no radioactivity was eluted with ribosomes. The amount of GDP bound (0.25 mol/mol of ribosome) was less than expected, as compared to the 60–70% of the ribosomes carrying Phe-tRNA^{Phe} bound enzymatically (24). This may depend both on low stability of the EF-Tu·GDP·ribosome complex and on the

TABLE 1. *Effect of kirromycin on EF-T activity in the GTPase reaction*

Additions	GTP hydrolyzed (pmol)					
	Without ribosomes		With ribosomes (6.7 pmol)		With ribosomes (20 pmol)	
	−Kirromycin	+Kirromycin	−Kirromycin	+Kirromycin	−Kirromycin	+Kirromycin
EF-T	0	4.5	0.1	24.3	0.2	55.5
EF-T + poly(U)	0.1	4.5	0.1	24.9	0.1	52.6
EF-T + Phe-tRNA^{Phe}	0	8.9	2.1	57.0	5.1	54.4
EF-T + Phe-tRNA^{Phe} + poly(U)	0.1	9.0	7.5	36.9	19.6	31.5
EF-T + tRNA^{Phe}	0.1	4.6	—	—	—	—
EF-T + Ac-Phe-tRNA^{Phe}	0.1	4.6	—	—	—	—

Sixteen pmol of EF-T, 210 pmol of [γ-^{32}P]GTP (800 Ci/mol), and, where indicated, 500 pmol of kirromycin, 2 μg of poly(U), 50 pmol tRNA^{Phe}, Ac-Phe-tRNA^{Phe}, or Phe-tRNA^{Phe}, and 6.7 or 20 pmol of ribosomes were incubated for 10 min at 30° in 60 μl of standard buffer and then assayed for GTP hydrolysis. —, not done.

TABLE 2. *Effect of kirromycin on EF-Tu activity in the GTPase reaction*

Additions	GTP hydrolyzed (pmol)			
	Without ribosomes and poly(U)		With ribosomes and poly(U)	
	−Kirromycin	+Kirromycin	−Kirromycin	+Kirromycin
EF-Ts	0	0.1	0.1	0.1
EF-Tu	0.1	3.6	0	33.3
EF-Tu + EF-Ts	0.1	4.4	0.2	36.4
EF-Tu + Phe-tRNA^{Phe}	0	5.5	5.4	17.1
EF-Tu + EF-Ts + Phe-tRNA^{Phe}	0.1	6.0	9.0	19.0

Two hundred pmol of [γ-^{32}P]GTP (800 Ci/mol) were incubated for 10 min at 30° in 75 μl of standard buffer as indicated with 22 pmol of EF-Ts, 17 pmol of EF-Tu, 30 pmol of Phe-tRNA^{Phe}, 500 pmol of kirromycin, and 20 pmol of ribosomes plus 2 μg of poly(U). After incubation, the samples were assayed for GTP hydrolysis.

TABLE 3. *Effect of kirromycin on Phe-tRNA^{Phe} binding to ribosomes*

Additions	[^{14}C]Phe-tRNA^{Phe} bound to ribosomes (pmol)	
	−Kirromycin	+Kirromycin
None	1.1	1.1
GTP	1.1	1.1
EF-T	1.1	3.5
EF-T + GTP	4.6	4.2

Ribosomes (125 pmol), 10 μg of poly(U), and 600 pmol of tRNA^{Phe} were incubated for 5 min at 30° in 500 μl of standard buffer and then chilled to 0°. Preincubation of ribosome with poly(U) and tRNA^{Phe} was performed to inhibit nonenzymatic binding of [^{14}C]Phe-tRNA^{Phe} (25). Standard buffer (15 μl) containing 30 pmol of [^{14}C]Phe-tRNA^{Phe} (513 Ci/mol) and, where indicated, 150 pmol of GTP, 30 pmol of EF-T, and 1 nmol of kirromycin was then added to 60 μl of the reaction mixture. After incubation for 5 min at 30°, binding of [^{14}C]Phe-tRNA^{Phe} to ribosomes was determined by filtration on nitrocellulose filters.

ability of EF-Tu to be retained on ribosomes in the presence of Phe-tRNA^{Phe} even without GTP (or GDP). This latter possibility is supported by the observation that, in the presence of kirromycin, EF-T-dependent binding of Phe-tRNA^{Phe} to ribosomes took place even in the absence of GTP (Table 3).

DISCUSSION

Kirromycin is a potent inhibitor of bacterial protein synthesis that selectively affects the reactions in which EF-Tu is involved. Up to now we have not been able to detect other effects except for a slight, and likely unspecific, inhibition of the EF-G-dependent ribosomal GTPase at high concentrations of the antibiotic.

The sequence of reactions that EF-Tu undergoes during each round of polypeptide elongation has been elucidated in the past few years (for a review, see ref. 26). EF-Tu interacts with GTP and aminoacyl-tRNA and catalyzes the enzymatic binding of aminoacyl-tRNA to ribosomes with concomitant GTP hydrolysis. This is followed by the release of EF-Tu·GDP complex from ribosomes, which allows the transfer of the peptidyl moiety from peptidyl-tRNA to the newly bound aminoacyl-tRNA (22, 23). EF-Ts has so far been implicated only in the exchange of EF-Tu-bound GDP with GTP, allowing the recycling of EF-Tu (12). In bacterial cell extracts, EF-Tu strongly associates with EF-Ts, forming the stable complex EF-T, which can be purified to homogeneity (10).

Our results show that kirromycin does not prevent the enzymatic binding of aminoacyl-tRNA to ribosomes but inhibits subsequent peptide bond formation. This inhibition is not caused by blockage of the peptidyl transferase activity of ribosomes, as indicated by the occurrence of peptide bond formation with puromycin or with nonenzymatically bound aminoacyl-tRNA, but appears to be related to the failure of EF-Tu to be released from the ribosome after GTP hydrolysis. A second effect of the antibiotic is the uncoupling of GTP hydrolysis from the binding of aminoacyl-tRNA to ribosomes. Kirromycin enables EF-Tu to catalyze GTP hydrolysis even in the absence of ribosomes and aminoacyl-tRNA. This reaction conserves several features associated with the one occurring upon binding of aminoacyl-tRNA to ribosomes in the absence of the antibiotic: it is selectively stimulated by aminoacyl-tRNA but not by uncharged tRNA or *N*-acylated aminoacyl-tRNA, and its K_m for GTP is the same as that of the aminoacyl-tRNA- and ribosome-dependent EF-T GTPase.

Since ribosomes are needed for EF-Tu-dependent GTPase activity, it has not been clear whether the catalytic center for the hydrolysis of the γ-phosphate of GTP is situated on the elongation factor or on the ribosome. The appearance of the kirromycin-dependent GTPase reaction with EF-Tu alone indicates that this center is located primarily on the elongation factor. However, the important role of the ribosomes in this reaction is made clear by the fact that they can stimulate the kirromycin-induced EF-Tu GTPase more than 10-fold.

Kirromycin is able to enhance the formation of the EF-Tu-GTP complex, to stabilize the binding of EF-Tu to ribosomes, and to promote the interaction of aminoacyl-tRNA with EF-Tu in the absence of GTP. Preliminary observations indicate that this antibiotic also causes a partial dissociation of EF-T into EF-Tu and EF-Ts and that this effect is strengthened by GDP. Its unique ability to mimic the action of GTP, aminoacyl-tRNA, or ribosomes on EF-Tu and to stimulate at the same time the interaction of each of these components with the elongation factor is striking. We conclude that kirromycin deeply affects the conformation of the EF-Tu molecule, reducing allosteric control between the sites responsible for interactions with other components of protein synthesis and rendering them, in general, more accessible.

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