IDENTIFICATION OF THE PART OF KIRROMYCIN STRUCTURE THAT ACTS ON ELONGATION FACTOR Tu

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1. Introduction

Some antibiotics inhibit protein biosynthesis in bacteria by acting on elongation factor Tu (EF-Tu) · kirromycin (mocimycin) blocks peptide chain elongation by preventing the release of EF-Tu from the ribosome [1–3], while pulvomycin (labilomycin) inhibits the enzymatic binding of aminoacyl-tRNA (aa-tRNA) to ribosomes by hindering the formation of aa-tRNA · EF-Tu · GTP complex [4].

The structure of pulvomycin is similar to that of the 5′-substituent of the central tetrahydrofurane moiety of kirromycin (see fig.1) and preliminary results in [4] indicate that both antibiotics bind to the same site of EF-Tu. Moreover, hydrolysis of the amide bond located in the 5′-substituent of the tetrahydrofurane moiety of kirromycin produces two fragments [5] which are completely inactive on EF-Tu [3]. These observations suggest that this part of kirromycin structure could be responsible for the action on EF-Tu.

Periodate oxidation of mocimycin (kirromycin) results in the formation of an aldehyde which was purified as its 2,4-dinitrophenylhydrazone [5]. The fragment, named fragment 7, virtually corresponds to the entire 5′-substituent of the central tetrahydrofurane moiety of kirromycin (fig.1).

Here, I have investigated the activity of fragment 7 in the in vitro system of *Escherichia coli* and found that this fragment can promote all the effects of the intact antibiotic on EF-Tu reactions.

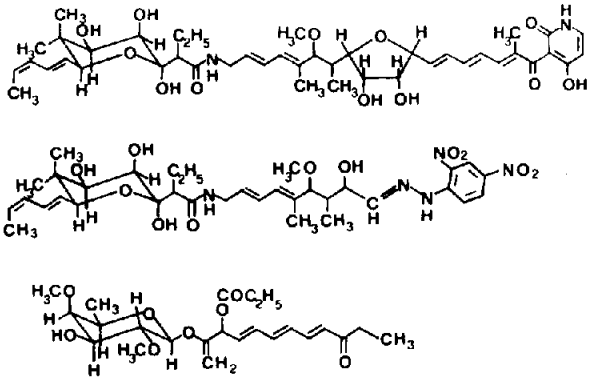

Fig.1. Structure of kirromycin (mocimycin), fragment 7 and pulvomycin (from top to bottom). The 5′-and 2′-substituents of the tetrahydrofurane moiety of kirromycin correspond to the parts of the antibiotic structure that are located in the figure at the left and at the right side of the tetrahydrofurane ring, respectively.

2. Materials and methods

2.1. *Antibiotics*

Kirromycin was a gift of Dr H. Wolf, University of Tubingen and fragment 7 was kindly donated by Dr C. Vos. Research Labs. of Gist-Brocades, Delft. The high purity of fragment 7 was confirmed by thin-layer chromatography analysis on precoated plates (silica gel 60 F-254, Merck, Darmstadt). Development of the chromatograms with 3 different solvent systems resulted in the migration of the product as a single spot of UV-absorbing material without evidence of contaminants. Moreover, the in vitro biological activity of the fragment was not affected by its reisolation by preparative thin-layer chromatography on silica gel with the solvent system chloroform–methanol–ethanol (48:1:1). This ruled out the possibility that fragment 7 (R_F 0.48) could be trace-contaminated by kirromycin (R_F 0.10) or by kirromycin 2,4-dinitrophenylhydrazone (R_F 0.19).

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2.2. *Other materials*

NH_4Cl-washed ribosomes, homogeneous elongation factor G (EF-G) and T (EF-T, the 1:1 complex of elongation factors Tu and Ts), crystalline EF-Tu · GDP and phenylalanyl-tRNA synthetase, all from *E. coli* B, were prepared as reported [2,6–8]. EF-Tu (90–95% GDP-free) was obtained by EDTA treatment of EF-Tu · GDP according to [9]. [^{14}C]Phe-tRNA$^{Phe}_{coli}$ (65% pure, 487 Ci/mol) and [γ-^{32}P]GTP were prepared as in [10,11].

2.3. *Assays*

All reactions were carried out in TNM 7 buffer (40 mM Tris–HCl, pH 7.8; 50 mM NH_4Cl; 7 mM $MgCl_2$; 2 mM dithiothreitol). Reaction mixtures also contained 0.3–1.5% ethanol carried over with the antibiotics. In each experiment the concentration of ethanol was kept constant in all samples including the control without antibiotics. Assays of poly(phenylalanine) synthesis, GTP hydrolysis, binding of Phe-tRNA to poly(U) · ribosomes and formation of EF-Tu · GTP and Phe-tRNA · EF-Tu · GTP complexes were performed as in [1–3,8,12]. Details of the experimental conditions are given in the legends to table and figures.

3. Results

3.1. *Poly(U)-directed poly(phenylalanine) synthesis*

As illustrated in fig.2, fragment 7 inhibited poly(phenylalanine) synthesis in the in vitro system of *E. coli*. The inhibition of the polymerization reaction by the fragment required its use at concentrations much higher than those needed by the intact antibiotic. As judged from the respective concentrations causing 50% inhibition (10^{-4} M and 2.3×10^{-7} M) the affinity of fragment 7 for EF-Tu was lower than that of kirromycin by nearly 3 orders of magnitude. Because of its poor solubility, fragment 7 could be tested only up to 0.3 mM at which the elongation reaction was 80% inhibited.

3.2. *EF-Tu-dependent GTPase activity*

The hydrolysis of GTP that takes place during the enzymatic binding of aa-tRNA to ribosomes requires EF-Tu, aa-tRNA, ribosomes and the appropriate messenger RNA [13]. In the presence of kirromycin, EF-Tu can catalyze the reaction even in the absence of the other components [1–3]. This kirromycin-induced GTPase activity of EF-Tu is low at the ionic conditions used in this work, but it can be stimulated up to 3- and 15-fold by the addition of Phe-tRNA and ribosomes, respectively [1–3,14]. By contrast, pulvomycin inhibits the reaction even in the presence of all components [4].

Fragment 7 was found to have the same effect of kirromycin on EF-Tu GTPase activity. This is illustrated in fig.3 for the system containing EF-Tu and ribosomes. Also in this case, the maximal activity of the intact antibiotic was not reached by fragment 7 because of its low solubility and poor affinity for EF-Tu.

Results equivalent to those depicted in fig.3 were

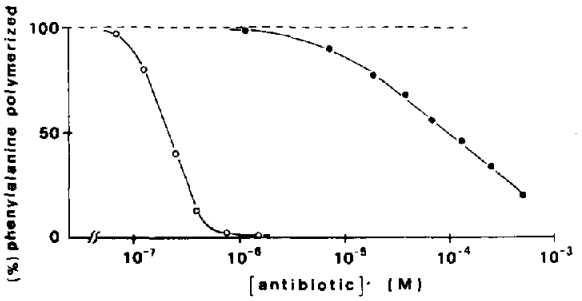

Fig.2. Inhibition of poly(phenylalanine) synthesis by fragment 7 and kirromycin. Reaction mixtures contained in 75 μl TNM 7 buffer 25 pmol ribosomes, 5 μg poly(U), 35 pmol EF-G, 9 pmol EF-T, 10 pmol phenylalanyl-tRNA synthetase, 60 pmol tRNA^{Phe}, 180 pmol [^{14}C]phenylalanine (486 Ci/mol), 75 nmol ATP and 15 nmol GTP. Reaction was for 10 min at 30°C. Samples were analyzed for phenylalanine polymerized as in [8]. Values are given as % of the amount of phenylalanine incorporated in the control without antibiotics (32.5 pmol): plus kirromycin (○); plus fragment 7 (●).

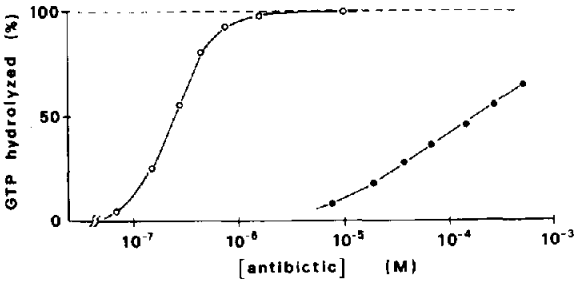

Fig.3. Activation of uncoupled EF-Tu GTPase activity by fragment 7 and kirromycin. Reaction mixtures contained in 75 μl TNM 7 buffer 40 pmol ribosomes, 9 pmol EF-T, 310 pmol [γ-^{32}P]GTP (510 Ci/mol) and kirromycin (○) or fragment 7 (●). After 15 min at 30°C, samples were analyzed for $^{32}P_i$ liberated [7]. Values are given as % of the amount of GTP hydrolyzed in the presence of the optimal concentration of kirromycin (41.5 pmol). In the absence of antibiotics the amount of GTP hydrolyzed was 0.3 pmol.

obtained in the systems containing EF-Tu alone or EF-Tu plus Phe-tRNA (not shown).

3.3. *Interaction of EF-Tu with GTP and aminoacyl-tRNA*

In the absence of aa-tRNA the affinity of EF-Tu for GDP is almost 3 orders of magnitude higher than that for GTP [9,13]. Kirromycin and pulvomycin dramatically increase the affinity of EF-Tu for GTP: in their presence free GTP can displace GDP bound to EF-Tu [1,2,9]. Fragment 7 was also found to promote the GTP–GDP exchange reaction (fig.4). At 0.1 mM the fragment caused 44% of the exchange observed in the presence of 10 μM kirromycin, an effect equivalent to that observed in the induction of EF-Tu GTPase (see fig.3). Moreover, fragment 7 was found to stimulate the exchange of free GDP with EF-Tu-bound GDP like kirromycin [9].

The EF-Tu · GTP complexes formed in the presence or the absence of kirromycin, but not that formed in the presence of pulvomycin, bind aa-tRNA [1,2,4,9,13]. The EF-Tu · GTP complex formed in the presence of fragment 7 is able to bind aa-tRNA as indicated by its release from nitrocellulose filters upon addition of Phe-tRNA (fig.5) [12].

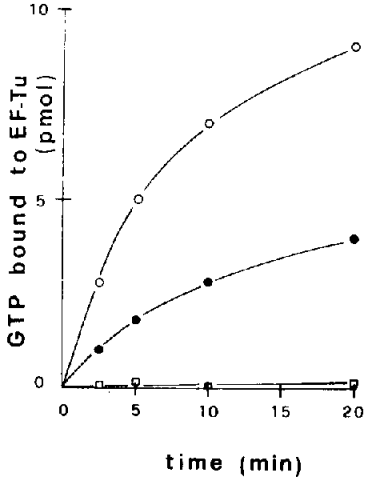

Fig.4. Effect of fragment 7 and kirromycin on the exchange of free GTP with GDP bound to EF-Tu. Reaction mixtures contained in 0.4 mol TNM 7 buffer 80 pmol EF-Tu · GDP, 250 pmol [γ-^{32}P]GTP (360 Ci/mol) and kirromycin or fragment 7 as indicated. The exchange reaction was carried out at 0°C and started by the addition of GTP. At the indicated times, the amount of EF-Tu · GTP complex formed was determined on 90 μl aliquots [1]: without antibiotics (□); with 10 μM kirromycin (○); with 0.1 mM fragment 7 (●).

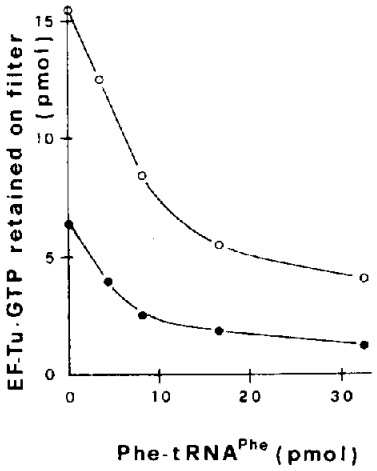

Fig.5. Ability of EF-Tu · GTP complexes formed in the presence of fragment 7 and kirromycin to bind Phe-tRNA. EF-Tu · GTP complexes were prepared by incubation of 200 pmol EF-Tu · GDP with 610 pmol [γ-^{32}P]GTP (330 Ci/mol) in the presence of 15 μM kirromycin (○) or 0.15 mM fragment 7 (●) for 30 min at 24°C and in a final volume of 0.52 ml TNM 7 buffer. Then, 80 μl aliquots of the two reaction mixtures were added to 40 μl TNM 7 buffer containing Phe-tRNA as indicated. After 10 min at 0°C samples were analyzed for the amount of EF-Tu · GTP complex retained on nitrocellulose filters (millipore HAPW 0.45 μm). The loss of EF-Tu · GTP complex from filter measures the amount of Phe-tRNA · EF-Tu complex formed.

3.4. *EF-Tu-directed binding of aminoacyl-tRNA to ribosomes*

In the presence of kirromycin, EF-Tu can promote the binding of aa-tRNA to the ribosomal A site even without GTP, while pulvomycin completely blocks the reaction [1,3,4].

EF-Tu and EF-Tu · GDP became able to promote the binding of Phe-tRNA to poly(U) · ribosomes in the presence of 0.1 mM fragment 7 (table 1). Little stimulation of the reaction was observed with EF-T (EF-Tu · EF-Ts complex). This indicates that EF-Ts interferes with the binding of fragment 7 to EF-Tu. A competition between kirromycin and EF-Ts for the interaction with EF-Tu has been observed [2,15]. In the experiment of table 1, kirromycin could stimulate the binding reaction with EF-T because the antibiotic was used at a concentration at which it can displace EF-Ts from EF-Tu.

Table 1
Effect of fragment 7 and kirromycin on the EF-Tu-directed binding of Phe-tRNA to poly(U) · ribosomes

Experimental conditions	[^{14}C]Phe-tRNA bound to ribosomes (pmol)		
	Control	+ Fragment 7	+ Kirromycin
+ EF-Tu	0.1	2.5	4.6
+ EF-Tu · GDP	0.2	2.6	4.8
+ EF-Tu · EF-Ts	0.0	0.5	4.3
+ EF-Tu · EF-Ts + GTP	5.8	5.2	5.1

Reaction mixtures contained 25 pmol ribosomes, 5 μg poly(U), 54 pmol $tRNA^{Phe}$, 12 pmol [^{14}C]Phe-tRNA and, when indicated, 40 pmol EF-Tu, EF-T or EF-Tu · GDP, 0.2 nmol GTP, 7.5 nmol fragment 7 or 0.75 nmol kirromycin. Ribosomes were preincubated with poly(U) and $tRNA^{Phe}$ in order to fill the P site prior to the addition of the other components [1]. The reaction mixtures (75 μl) in TNM buffer containing 10 mM $MgCl_2$ were incubated for 10 min at 30°C and then analyzed for the amount of Phe-tRNA bound to ribosomes [1]. All values are corrected for the amount of Phe-tRNA bound non-enzymatically to ribosomes in the absence of EF-Tu (2.1 pmol)

4. Discussion

The ability of fragment 7 to promote all the effects of kirromycin indicates that only the part of the antibiotic structure which corresponds to the 5′-substituent of the central tetrahydrofurane moiety is directly responsible for the action on EF-Tu. The following observations suggest that the main and possibly exclusive role of the rest of the antibiotic structure is to increase the antibiotic affinity for the elongation factor:

(i) Fragment *4a* of mocimycin (kirromycin) which contains both the tetrahydrofurane ring and its 2′-substituent [5] is completely inactive [3];
(ii) Chemical modifications of the acid hydroxyl and cheto functions located in the 2′-substituent do not affect the mode of action of kirromycin, but lower its affinity for EF-Tu (G. C., unpublished);
(iii) Fragment 7 interacts very weakly with EF-Tu (in this case, however, the presence of the 2,4-dinitrophenylhydrazone group could also contribute to reduce the affinity of the fragment for EF-Tu).

The finding that kirromycin acts on EF-Tu with the part of its structure which is related to pulvomycin and the results in [4] indicating that these antibiotics compete for the interaction with EF-Tu suggest a common site of action on the elongation factor. If this assumption is correct, the distinct effects of kirromycin and pulvomycin on EF-Tu activity should depend on the ability of these antibiotics to promote different conformational changes of the same EF-Tu site presumably as a consequence of the differences between the structure of pulvomycin and that of the 5′-substituent of the tetrahydrofurane moiety of kirromycin.

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