HYDROLYSIS OF GTP BY THE ELONGATION FACTOR Tu·KIRROMYCIN COMPLEX

Specific action of monovalent cations

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1. Introduction

The antibiotic kirromycin binds to elongation factor Tu (EF-Tu) in a 1:1 molar ratio and induces a GTP hydrolysis in the absence of ribosomes, aa-tRNA and elongation factor Ts(EF-Ts) with the characteristics of a turnover reaction [1–4]. That kirromycin can trigger the GTP hydrolysis with EF-Tu alone implies that the catalytic center of this reaction is located on the elongation factor and not on the ribosome, as had been previously suggested [5]. It is however important to note that ribosomes and aa-tRNA, individually or in combination, both stimulate the kirromycin-induced activity. The ability of a small molecule, like kirromycin, to complement and/or substitute physiological effectors, like ribosomes, aa-tRNA and EF-Ts, offers a rare opportunity to study regulation mechanisms in a complex enzyme system.

In this article, we show for the first time that the kirromycin-activated EF-Tu GTPase is absolutely dependent on monovalent cations. Ribosomes, and partially also aa-tRNA, abolish this requirement.

2. Materials and methods

2.1. *Materials*

Electrophoretically homogeneous EF-Tu, 0.5 M NH_4Cl-washed ribosomes, Phe-tRNA^{Phe} (all from *Escherichia coli* A19 or B) and the other materials were as in [1–4,6].

2.2. *Assays*

2.2.1. GTPase reaction

The 75 μl reaction mixtures contained 20 mM Tris–HCl (pH 7.5 at 20°C), 10 mM $MgCl_2$, 20 pmol EF-Tu·GDP, 50 μM kirromycin and 10 μM [γ-^{32}P]GTP (spec. act. 280 cpm/pmol), with or without 40 pmol 70 S ribosomes and/or 100 pmol Phe-tRNA^{Phe}; no poly(U) was added to avoid blockage of EF-Tu on the ribosome [3]. In the absence of the antibiotic, 5 μg poly(U) as well as 10 pmol EF-Ts were added, the latter to keep the reaction linear after the first round of GTP hydrolysis [4]. Incubations were carried out at 30°C, for 15 min in the presence of kirromycin and for 5 min in its absence. In the intervals of time reported, the kinetics were linear, with or without kirromycin. Activity was measured as the liberation of $^{32}P_i$ [6].

2.2.2. Exchange of EF-Tu-bound GDP with free [γ-^{32}P]GTP

Incubations were carried out at 0°C in 900 μl reaction mixtures containing 20 mM Tris–HCl (pH 7.5), 7 mM $MgCl_2$, NH_4Cl as indicated, 63 pmol EF-Tu·GDP, 60 pmol GDP and 670 pmol [γ-^{32}P]GTP (the latter added at zero time), plus or minus 50 μM kirromycin. At the times indicated, 100 μl aliquots were withdrawn, and the radioactivity retained on

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nitrocellulose filters (Sartorius SM 11306) was measured [4].

2.2.3. Determination of the equilibrium constants

Equilibrium constants of the EF-Tu·kirromycin complex for GTP or GDP were calculated from the apparent association and dissociation rate constants at 0°C, to avoid the hydrolysis of GTP [4]. Kirromycin was added to 50 μM, a concentration inducing maximal EF-Tu GTPase activity also at high concentrations of NH_4^+Cl (unpublished result), very probably by converting all the EF-Tu into EF-Tu·kirromycin [4].

3. Results

3.1. *Kirromycin-induced GTPase activity in the absence of ribosomes*

Figure 1 shows the effect, at 10 mM Mg^{2+}, of increasing $[NH_4^+]$ on the EF-Tu GTPase activity induced by kirromycin in the presence (upper curve) and absence (lower curve) of Phe-$tRNA^{Phe}$. Without monovalent cations, activity in the absence of Phe-$tRNA^{Phe}$ was close to zero, while in its presence the GTPase activity rose to about 10% of the maximum. Addition of NH_4^+ dramatically increases the activity, in both cases reaching a similar level; with Phe-$tRNA^{Phe}$ this occurs abruptly. As a result, stimulation by Phe-$tRNA^{Phe}$ decreases with rising $[NH_4^+]$.

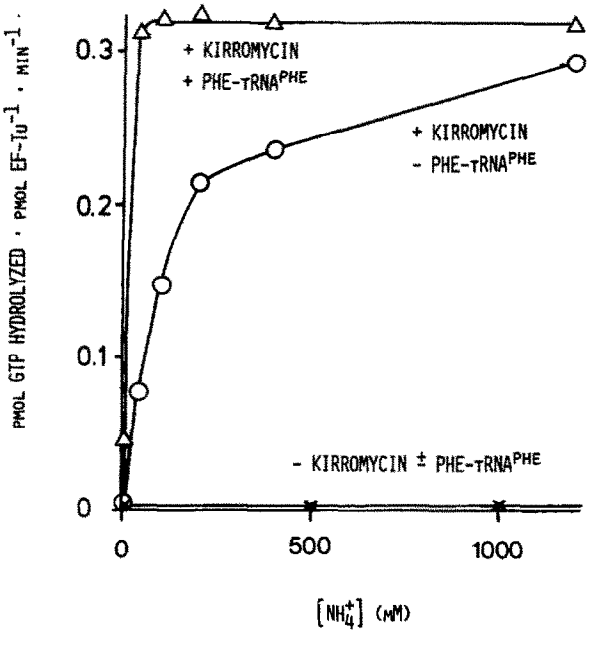

Fig.1. Dependence on NH_4^+ of the EF-Tu·kirromycin GTPase activity in the absence of ribosomes. For experimental details see section 2. (○) EF-Tu with kirromycin alone; (△) EF-Tu with kirromycin plus Phe-$tRNA^{Phe}$; (×) EF-Tu without kirromycin, with or without Phe-$tRNA^{Phe}$. At high salt the kirromycin-induced GTPase activity displayed a turnover of 3–4 pmol GTP hydrolyzed/pmol EF-Tu in 15 min.

3.2. *EF-Tu-dependent GTPase activity in the presence of ribosomes, with or without kirromycin*

In this case no dependence on NH_4^+ was observed. With kirromycin in the absence of Phe-$tRNA^{Phe}$, after a slight increase in activity between 0.4 mM and 100 mM NH_4^+, there is a steady decrease, with activity at 1.2 M NH_4^+ approaching that observed in the absence of ribosomes (fig.2, dashed line). Consequently, stimulation by ribosomes is from very great in the lower range of $[NH_4^+]$ to negligible at higher $[NH_4^+]$. Preliminary results suggest that this reduction of stimulation cannot only be explained by the dissociation of the 70 S ribosomes. The additional presence of Phe-$tRNA^{Phe}$ further stimulates the GTPase activity by 20–50% and shifts the NH_4^+ optimum to about 400 mM. For the physiological EF-Tu GTPase system without kirromycin (fig.2), the dependence on NH_4^+ is the mirror image of the pattern observed with EF-Tu alone in the presence of the antibiotic: maximum activity is found between 0.4 mM and 40 mM NH_4^+, and is strongly inhibited by increasing NH_4^+.

We have also observed that both Na^+ and K^+ display similar qualitative effects to NH_4^+ in the experiments reported in this section and in section 3.1., unlike divalent cations and monovalent anions (not illustrated).

3.3. *Exchange of GDP bound to EF-Tu·kirromycin with free [γ-^{32}P]GTP*

Kirromycin increases the affinity of EF-Tu for GTP, achieving a binding constant similar to that for GDP [2,4,7,8], which is normally lower by 2 orders of magnitude [4,9,10]. This is accompanied by increased rates of EF-Tu·GDP association and dissociation [4,7,11]. These effects, by allowing a rapid exchange of the reaction product, GDP, with GTP, appear to be important for the turnover GTPase activity of the EF-Tu·kirromycin complex. We have

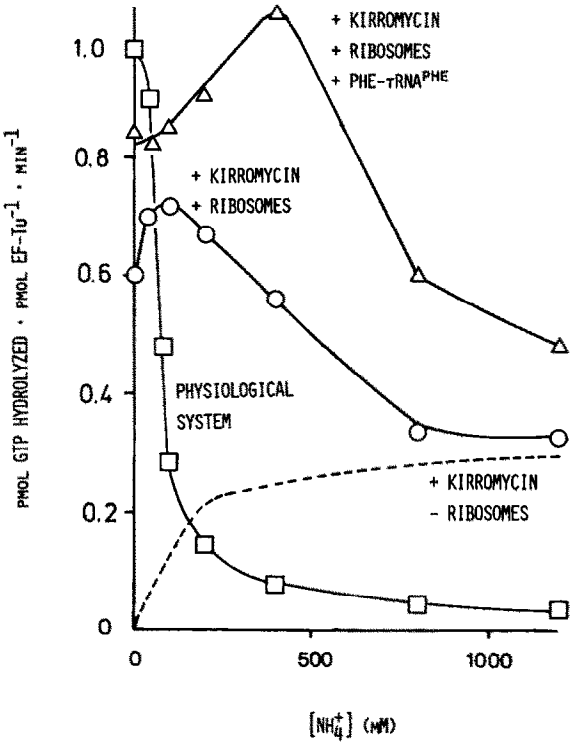

Fig.2. Independence of NH_4^+ of the EF-Tu·kirromycin GTPase activity in the presence of ribosomes: comparison with the ribosome-free and the physiological system. For experimental conditions see section 2. (○) EF-Tu with kirromycin and ribosomes; (△) EF-Tu with kirromycin and ribosomes plus Phe-tRNA^{Phe}; (□) EF-Tu with ribosomes·poly(U), Phe-tRNA^{Phe} and EF-Ts in the absence of kirromycin; (– – – –), activity in the presence of EF-Tu and kirromycin without ribosomes, taken from fig.1. The assays done in the presence of ribosomes contained 0.4 mM NH_4^+ carried over with the ribosomes.

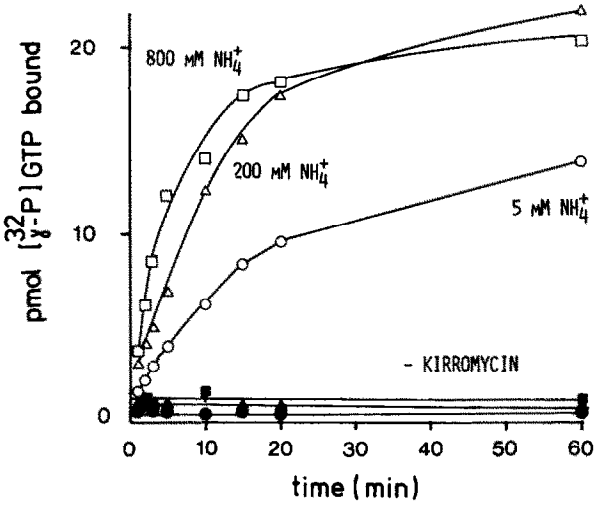

Fig.3. Kinetics of the exchange of GDP bound to EF-Tu with free GTP. For conditions see section 2. (○) 5 mM NH_4^+, (△) 200 mM NH_4^+, (□) 800 mM NH_4^+, all with 50 μM kirromycin; (●) 5 mM NH_4^+, (▲) 200 mM NH_4^+, (■) 800 mM NH_4^+, all without kirromycin.

examined the effect of monovalent cations on the EF-Tu·GDP–GTP exchange rate (fig.3), and find only a modest stimulation by NH_4^+ between 5 mM and 200 mM, the range most strongly affecting the GTPase activity.

This is also mirrored in the equilibrium constants between the EF-Tu·kirromycin complex and GTP or GDP (table 1). Because of the similar increase found for both reactions with increasing [NH_4^+], the affinity of the EF-Tu·kirromycin complex for GTP relative to that for GDP (lowermost line of table 1) shows only a small change.

4. Discussion

The importance of the ionic environment in the expression of the EF-Tu GTPase activity is evident from the present results. The inability of divalent cations to substitute for the monovalent ones suggests a specific effect which cannot be explained simply in terms of ionic strength. In the absence of kirromycin the physiological effectors, aa-tRNA and ribosomes,

Table 1
Equilibrium constants of the kirromycin·EF-Tu·GTP and kirromycin·EF-Tu·GDP complexes at different NH_4^+ concentrations

	[NH_4^+] (mM)		
	3	200	800
K'_{GTP}[a]	0.5	1.3	5.5
K'_{GDP}[a]	1	3.5	19
K'_{GTP}/K'_{GDP}	0.50	0.37	0.29

[a] The K' values are given in nM

The K' was determined from the apparent association and dissociation rate constants at 0°C as in [4], at a 50 μM kirromycin concentration

need no or little monovalent cations; at relatively low concentration these even become inhibitory. By contrast, the GTPase activity of the EF-Tu·kirromycin complex shows a requirement for monovalent cations which appear to be closely involved in the GTP cleavage. Though NH_4^+ appears to accelerate the regeneration rate of the EF-Tu·GTP complex (see table 1), our results rule out the modification of the accessibility of the EF-Tu binding site for GTP and GDP as cause for the increased rate of GTP hydrolysis. Indeed, at 3 mM NH_4^+, where the GTPase activity is close to zero, kirromycin conserves the ability to lower the equilibrium constant between EF-Tu and GTP to values close to those observed for EF-Tu·GDP [2,4]. Moreover, in experiments not illustrated we have determined that, with kirromycin, in the whole range of $[NH_4^+]$ the dissociation rate of EF-Tu·GDP, which without kirromycin is the slowest reaction in the regeneration of EF-Tu·GTP [4], becomes much faster than the GTPase activity. We conclude that the stimulation of the kirromycin-induced activity by NH_4^+ is due to a direct effect on the hydrolytic process.

Phe-tRNA^{Phe} seems to induce a more stable conformation of the catalytic center: beyond a critical NH_4^+ concentration of about 40 mM, the GTPase activity remains constant. The ribosome seems to relieve the need for monovalent cations, perhaps by the importation of bound ions. The actions of aa-tRNA and ribosomes are additive, showing that each of the two acts directly and independently on the catalytic center of EF-Tu. In their turn, the monovalent cations can substitute for the physiological effectors inducing a microenvironment conducive to the cleavage of GTP.

We have reported [1–4], a 2–3-fold stimulation of the kirromycin-induced EF-Tu GTPase activity by aa-tRNA, and 5–20-fold stimulation by ribosomes, with an additional increase in the presence of both effectors. Here we show that the stimulatory action of aa-tRNA and ribosomes is a function of the monovalent cation concentration and that at near-physiological concentration (200 mM, [12]), the level of EF-Tu GTPase activity induced by kirromycin with EF-TU alone is somewhat higher than that induced by the physiological effectors in the absence of the antibiotic. Lower and higher concentrations lead to a large difference between the two systems.

The interdependence between the effect of the monovalent cations and those of the other components of the system open interesting possibilities for studying the reaction mechanisms, particularly in view of the theory of ionic regulation proposed [13]. To this end, we are currently investigating the modulation of the EF-Tu GTPase activity by other ionic parameters, such as pH and divalent cations.

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