THE GTPase ACTIVITY OF ELONGATION FACTOR Tu AND THE 3′-TERMINAL END OF AMINOACYL-tRNA

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1. Introduction

In the elongation cycle, the binding of the ternary complex EF-Tu · GTP · aa-tRNA to the mRNA · ribosome complex is accompanied by the hydrolysis of GTP, a prerequisite for the release of EF-Tu from the ribosome (review [1]). We have utilized the antibiotic kirromycin to analyze the role of the individual components of the EF-Tu-dependent GTPase reaction, since this compound allows EF-Tu to support a turnover of GTPase activity which is specifically stimulated by aa-tRNA and ribosomes [2–6]. As in the physiological system, only aminoacylated tRNA and not deacylated tRNA or *N*-acetylaminoacyl-tRNA displays an effect in the kirromycin-dependent activity. Kirromycin can be therefore a useful tool to study the involvement of the 3′-terminal end of aa-tRNA in the GTPase activity of EF-Tu.

Here, we show that 3′-terminal aa-tRNA fragments, containing 1–5 residues, can stimulate the GTPase activity of EF-Tu induced by kirromycin. The effect, which occurs already with 2′(3′)-*O*-L-aminoacyladenosine, is increased by the presence of the other residues, the penultimate one playing a critical role. Ribosomes enhance the sensitivity of the system.

Abbreviations: aa-tRNA, aminoacyl-tRNA; $tRNA^{Phe}$, phenylalanine specific tRNA; $tRNA_1^{Val}$, isoacceptor 1 of valine specific tRNA; A-Phe, 2′(3′)-*O*-L-phenylalanyladenosine; A-Val, 2′(3′)-*O*-L-valyladenosine; CA-Phe, cytidylyl-(3′-5′)-2′(3′)-*O*-L-phenylalanyladenosine; CCA-Phe, CACCA-Phe, CA-Val, CCA-Val, other aminoacylated fragments derived from the 3′-terminal end of Phe-$tRNA^{Phe}$ and Val-$tRNA_1^{Val}$; EF-Tu, elongation factor Tu

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2. Materials and methods

All biological components, materials and methods not quoted in this section were as in [2–8]. Pure EF-Tu and NH_4Cl-washed ribosomes were from *Escherichia coli* MRE 600. Kirromycin (= mocimycin, [5]) was obtained from Dr H. Wolf (University of Tübingen) and Dr R. Beukers (Gist-Brocades, Delft). Cytosine-3′-monophosphate was from Boehringer and puromycin from Sigma or Serva. The $tRNA^{Phe}$ (25–100% pure) and $tRNA_1^{Val}$ (25–50% pure, a gift of Dr G. Sander) were isolated from total tRNA (Schwarz/Mann) by chromatography on BD-cellulose and Sepharose 4B (Pharmacia) [9,10]. The various 3′-terminal fragments (CACCA-Phe, CCA-Phe, CA-Phe, A-Phe, CCA-Val, CA-Val and A-Val) were obtained from [^{14}C]Phe-$tRNA^{Phe}$ (spec. act. 50 cpm/pmol) and [^{14}C]Val-$tRNA_1^{Val}$ (spec. act. 50 cpm/pmol) according to known methods of enzymatic digestion with RNase T_1 (Calbiochem), RNase U_2, calf spleen phosphodiesterase and pancreatic RNase (all from Boehringer) [11,12]. They were purified by chromatography on DEAE–Sephadex A-25 (Pharmacia) at pH 4.5, followed by chromatography on SP–Sephadex C-25 (Pharmacia) at pH 3.5. Elutions were carried out with a gradient of ammonium acetate. For CA-Phe and CA-Val an additional electrophoretic step on Whatmann 3 MM paper at pH 3.5 and 50 V/cm (Gilson Electrophorator) was employed. Purity of the fragments, calculated spectrophotometrically and by specific activity, was 90–100%. The fragments were ≥95% aminoacylated. After lyophilisation, the fragments were dissolved in 2 mM acetic acid and kept at −70°C. Particular care was taken to avoid the presence of monovalent cations in the final solution, since they greatly influence the EF-Tu · kirromycin GTPase activity [7].

To increase the stimulatory effect of aa-tRNA, in the absence of ribosomes we have worked at 25 mM NH_4Cl while the $MgCl_2$ concentration, which is not critical, was 10 mM. Stimulation by Phe-tRNA was ~7-fold. Since in these ionic conditions ribosomes enhance the GTPase activity of EF-Tu · kirromycin in the absence of aa-tRNA considerably [7], in their presence we have modified the ionic concentrations (5 mM NH_4Cl and 30 mM $MgCl_2$) to obtain a stronger stimulation by aa-tRNA (9–12-fold with Phe-$tRNA^{Phe}$) thanks to the lower background of GTPase activity. Under all conditions tested the GTP concentration was saturating and the reaction kinetics linear. Most assays were carried out in 10 μl final vol., the results being comparable with those obtained in 15 and 75 μl. The reaction was started with [γ-^{32}P]GTP (spec. act. 1500–4000 cpm/pmol). The non-hydrolyzed [γ-^{32}P]GTP was sequestered by addition of activated charcoal in 1 M HCl [13] and the mixture was centrifuged for 15 min at 5000 rev./min. An aliquot of the supernatant was added to Aquasol 2 (New England Nuclear) and the radioactivity of $^{32}P_i$ counted in an Intertechnique SL 4000 liquid scintillation spectrometer.

3. Results

To obtain some indications of the role of the amino acid residues in the interaction with EF-Tu, we have done our experiments with fragments derived from two different aa-tRNAs.

Fig.1 illustrates the effect of increasing concentrations of Phe-$tRNA^{Phe}$ (panel A) and Val-$tRNA_1^{Val}$ (panel B) and their 3′-terminal fragments on the rate of the EF-Tu-dependent GTP hydrolysis induced by kirromycin in the absence of ribosomes. Stimulation of the GTPase activity by CACCA-Phe, the longest aminoacyl-oligonucleotide used, was stronger than with CCA-Phe and A-Phe, the latter fragment being by far the least active. In this system CA-Phe was not tested. A similar pattern was observed with the 3′-terminal fragments derived from Val-$tRNA_1^{Val}$:CCA-Val and CA-Val were both as active as CCA-Phe while the effect of A-Val was as little as that of A-Phe. With both intact aa-tRNAs, stimulation was, however, much stronger than with the derived fragments.

Fig.2 illustrates the same series of experiments in the presence of ribosomes. Also in this system the intact aa-tRNA was the most effective. Panel A shows

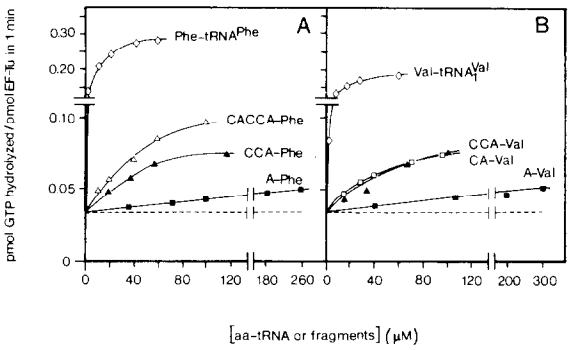

Fig.1. The rate of GTP hydrolysis catalyzed by EF-Tu in the presence of kirromycin as a function of the concentration of Phe-$tRNA^{Phe}$ (A), Val-$tRNA_1^{Val}$ (B) and their 3′-terminal fragments. The reaction mixture contained in 10 μl final vol.: 50 mM imidazolium acetate (pH 7.0); 25 mM NH_4Cl; 10 mM $MgCl_2$; 30 pmol [γ-^{32}P]GTP (spec. act. 3500 cpm/pmol); Phe-$tRNA^{Phe}$ (A) or Val-$tRNA_1^{Val}$ (B) and the respective 3′-terminal fragments as indicated; 50 μM kirromycin; 4 pmol EF-Tu. After 15 min incubation at 30°C the reaction was stopped with 50 μl 0.6 M $HClO_4$ and the $^{32}P_i$ liberated was measured as in section 2. (A) Phe-$tRNA^{Phe}$ (◇); CACCA-Phe (△); CCA-Phe (▲); A-Phe (■). (B) Val-$tRNA_1^{Val}$ (◇); CCA-Val (▲); CA-Val (□); A-Val (■).

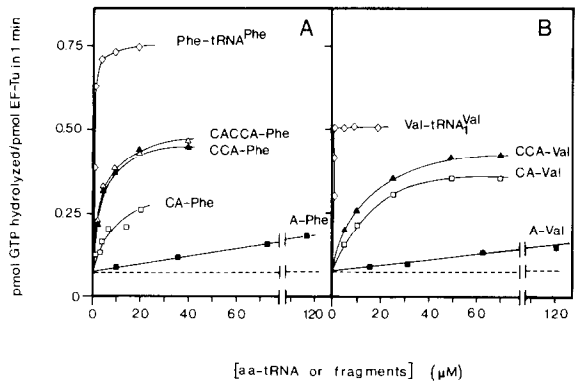

Fig.2. The rate of GTP hydrolysis catalyzed by EF-Tu in the presence of kirromycin and ribosomes as a function of the concentration of Phe-$tRNA^{Phe}$ (A), Val-$tRNA_1^{Val}$ (B) and their 3′-terminal fragments. The reaction mixture contained in 10 μl final vol.: 30 mM imidazolium acetate (pH 7.0); 5 mM NH_4Cl, 30 mM $MgCl_2$, 100 pmol [γ-^{32}P]GTP (spec. act. 1500 cpm/pmol), Phe-$tRNA^{Phe}$ (A) or Val-$tRNA_1^{Val}$ (B) and the respective fragments as indicated; 50 μM kirromycin; 4 pmol EF-Tu; 4 pmol 70 S ribosomes. After 15 min incubation at 30°C, the reaction was stopped with 50 μl 0.6 M $HClO_4$ and the $^{32}P_i$ liberated was measured as in section 2. (A) Phe-$tRNA^{Phe}$ (◇); CACCA-Phe (△); CCA-Phe (▲); CA-Phe (□); A-Phe (■). (B) Val-$tRNA_1^{Val}$ (◇); CCA-Val (▲); CA-Val (□); A-Val (■). (A,B) EF-Tu + kirromycin and ribosomes without additions (– –).

that CCA-Phe enhances the rate of GTP hydrolysis as well as CACCA-Phe. CA-Phe was 50% as effective, while A-Phe was again the least effective. Stimulation by CCA-Val reached almost that obtained with Val-tRNA$_1^{Val}$; CA-Val was somewhat less active than CCA-Val, while stimulation by A-Val was very small, being almost that of A-Phe.

In both systems, saturating concentrations of Phe-tRNA^{Phe} induced a 50% stronger stimulation than did Val-tRNA$_1^{Val}$. The difference in the stimulation by the intact molecules was not conserved in the magnitude of the effect for their derived fragments. Deacylated 3′-terminal fragments were inactive (not shown).

The effects described in fig.1 and 2 were evaluated by double reciprocal plots of the increment in GTPase activity *vs* the concentrations of 3′-terminal fragments or aa-tRNA. Table 1 reported the values obtained for the concentrations required for half-maximum stimulation of the rate of the GTP hydrolysis. These values can be considered to represent the apparent dissociation constant (K_d) of the aminoacylated 3′-terminal fragments or aa-tRNA from the EF-Tu · kirromycin complex. The required concentration for aa-tRNA is lower than for aminoacyladenosine by 2 orders of magnitude, while the difference is reduced to 1 order of magnitude only for the longer fragments. This already occurs with CA-Phe and CA-Val, indicating that the penultimate residue mostly contributes to the effect of the aminoacyl-oligonucleotides on the EF-Tu center for GTP hydrolysis. Ribosomes enhance the sensitivity of the system from 6–15-times, without greatly changing the general picture. The difference in stimulation between A-Phe or A-Val and the longer fragments was retained also at infinite concentrations (not shown).

Table 1
Concentration of Phe-tRNA^{Phe}, Val-tRNA$_1^{Val}$ and their 3′-terminal fragments inducing half-maximum stimulation of the EF-Tu GTPase activity in the presence of kirromycin

	−Ribosomes (μM)	+Ribosomes (μM)
Phe-tRNA^{Phe}	1.2	0.2
CACCA-Phe	80	6
CCA-Phe	100	6
CA-Phe	n.d.	10
A-Phe	500	75
Val-tRNA$_1^{Val}$	1.5	0.15
CCA-Val	100	12
CA-Val	100	17
A-Val	1000	140

The values represent the average of different experiments; n.d., not determined

The addition of cytosine 3′-monophosphate up to 1 mM did not increase the weak effect of A-Phe or A-Val either in the absence or in the presence of ribosomes (not shown).

We also investigated whether puromycin, an analogue of aminoacyladenosine, could stimulate the GTPase activity of EF-Tu in the presence of kirromycin. Neither in the presence, not in the absence of ribosomes were we able to observe any effect by using puromycin over 10 μM–5 mM (not shown).

4. Discussion

The use of kirromycin to activate the EF-Tu center for GTP hydrolysis facilitates the study of the regulation of this activity by aa-tRNA, ribosomes and cations [5]. Moreover, it permits to quantify precisely the effects, since in the presence of kirromycin the rate-limiting step of the turnover of the GTPase activity of EF-Tu is always GTP breakdown [6].

Our results show that 3′-terminal fragments of aa-tRNA are able to stimulate the catalytic activity of EF-Tu independently of the other domains of the aa-tRNA molecule. Though 2′(3′)-*O*-L-aminoacyladenosine alone can stimulate the EF-Tu GTPase activity, the presence of the penultimate residue appears to be critical for a productive interaction with EF-Tu. The observation that addition of cytosine 3′-monophosphate does not influence the effect of A-Phe or A-Val suggests that the cytidylic acid residue does not act directly on EF-Tu but affects the configuration of the aminoacyl-adenosine moiety [14]. The presence of other residues besides the penultimate residue appears to be less important.

Ribosomes, which strongly stimulate the catalytic activity of EF-Tu · kirromycin, appear to favor the activity of the 3′-terminal fragments even more than that of aa-tRNA. This observation as well as the size of the observed effects point to a prominent role of the 3′-terminal end in the interaction between aa-tRNA and EF-Tu. That the intact molecule is still more active than the derived fragments may depend either on additional interactions between other domains of the molecule and EF-Tu, or on a more specific posi-

tioning of the 3′-terminal end due to the intact acceptor stem.

Our observations agree well with [15], where in the presence of ribosomes 2′(3′)-*O*-L-aminoacyl-adenosine caused EF-Tu to support a GTPase activity. They are also consistent with results showing that CA-Phe is more effective than A-Phe in the protection of EF-Tu from binding of L-1-tosylamido-2-phenylethyl chloromethyl ketone, whereas puromycin has no effect [16].

The small differences between the stimulation by the fragments from Phe-tRNA^{Phe} and those from Val-tRNA$_I^{Val}$ indicate that in this case the nature of the amino acid does not exert a great influence on the reaction.

We feel that a systematic use of a sensitive system like the above will contribute to a better understanding of the role played by the various domains of the aa-tRNA molecule in the interaction with EF-Tu.

Note added in proof

After submission of the manuscript we learnt, P. Bhuta and S. Chládek had just reported (FEBS Lett. 122, 113–116, 1980) that in their system in the presence of aurodox (=methylated kirromycin) the GTPase activity of EF-Tu was enhanced to the same extent by both A-Phe and CA-Phe, and even more strongly by puromycin.

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