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Altered Regulation of the Guanosine 5'-Triphosphatase Activity in a Kirromycin-Resistant Elongation Factor Tu[†]

Ottavio Fasano and Andrea Parmeggiani*

ABSTRACT: In the preceding article a mutant elongation factor Tu (EF-Tu$_{D2216}$) resistant to the action of kirromycin was found to display a spontaneous guanosine 5'-triphosphatase (GTPase) activity, i.e., in the absence of aminoacyl transfer ribonucleic acid (tRNA) and ribosome·messenger RNA. This is the first example of an EF-Tu supporting GTPase activity in the absence of macromolecular effectors and/or kirromycin. In this study we show that this activity is elicited by increasing NH_4^+ concentrations. As additional effect, the mutation causes an increased affinity of EF-Tu for GTP. Ammonium dependence of the GTPase activity and increased affinity for GTP are two properties also found with wild-type EF-Tu in the presence of kirromycin [Fasano, O., Bruns, W., Crechet, J.-B., Sander, G., & Parmeggiani, A. (1978) *Eur. J. Biochem. 89*, 557–565; Sander, G., Okonek, M., Crechet, J.-B., Ivell, R., Bocchini, V., & Parmeggiani, A. (1979) *FEBS Lett. 98*, 111–114]. Therefore, both binding of kirromycin to wild-type EF-Tu and acquisition of kirromycin resistance introduce functionally related modifications. Kirromycin at high concentrations (0.1 mM) does not interact with mutant EF-Tu$_{D2216}$·GDP but still does with EF-Tu$_{D2216}$·GTP, in agreement with our previous finding that EF-Tu·GTP is the preferential target of the antibiotic in the wild type [Fasano, O., Bruns, W., Crechet, J.-B., Sander, G., & Parmeggiani, A. (1978) *Eur. J. Biochem. 89*, 557–565). The GTPase activity of mutant EF-Tu in the presence of aminoacyl-tRNA and ribosome·mRNA is much higher than with wild-type EF-Tu and also much less dependent on the presence of mRNA. Miscoding for leucine, measured as poly(U)-directed poly(phenylalanine/leucine) synthesis at increasing Mg^{2+} concentrations, is identical for both wild-type and mutant EF-Tu.

In the preceding article we have shown that kirromycin-resistant EF-Tu from the *Escherichia coli* strain D2216 is homogeneous and appears to display a GTPase[1] activity in the absence of the physiological effectors, aminoacyl-tRNA and ribosomes. Normally, with wild-type EF-Tu, these two components are required for the expression of the GTPase activity also when the reaction occurs in the absence of polypeptide synthesis (Gordon, 1969). Presence of mRNA on the ribosome is otherwise less strictly needed; Sander (1977) has shown that the GTPase activity can be uncoupled from mRNA by increasing the concentration of Mg^{2+}. Kirromycin induces a GTPase activity with wild-type EF-Tu in the absence of aminoacyl-tRNA and ribosomes; these two components individually or in combination enhance the GTPase activity induced by the antibiotic (Wolf et al., 1974, 1977; Parmeggiani et al., 1976) .

In this article we extend the study on the GTPase activity dependent on mutant EF-Tu. Our results show that the endogenous GTPase activity is a specific property of the mutant factor and is accompanied by a higher affinity for GTP. Remarkably, ammonium ions can influence this GTPase activity in a way resembling that on the kirromycin-induced GTPase of wild-type EF-Tu. The GTPase activity dependent on the mutant EF-Tu in the presence of aminoacyl-tRNA and mRNA·ribosomes appears to be less constrained by the codon–anticodon interaction.

Materials and Methods

Biological components, materials, and methods used and not described in this section or in the legends are the same as reported in the preceding paper.

Preparation of GDP-Free EF-Tu. GDP-free EF-Tu was obtained from crystalline EF-Tu·GDP purified from *E. Coli* B, D22, and D2216, as described before (Fasano et al., 1978) and was used within 15 min after its recovery from the column.

Determination of Association and Dissociation Rates between EF-Tu and GTP or GDP. The association rates of nucleotide-free EF-Tu with [γ-^{32}P]GTP or [^{3}H]GDP were measured by determining the apparent second-order rate constant of the reaction as described by Fasano et al. (1978). For the measurement of the dissociation rate of the EF-Tu·GTP and EF-Tu·GDP complexes, the apparent first-order rate constants were also determined as already reported (Fasano et al., 1978).

[1] Abbreviations used: EF-Tu, elongation factor Tu; EF-Ts, elongation factor Ts; EF-Tu$_{D22}$ and EF-Tu$_{D2216}$, elongation factor Tu from *E. coli* D22 and D2216, respectively; tRNA^{Phe}, phenylalanine-accepting transfer ribonucleic acid; enzyme·GTPase (EC 3.6.1.–); mRNA, messenger ribonucleic acid; GTP, guanosine 5'-triphosphate; GDP, guanosine 5'-diphosphate; GTPase, guanosine 5'-triphosphatase.
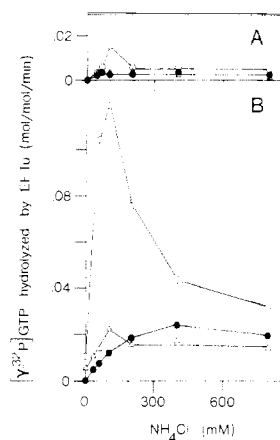
FIGURE 1: Effect of NH_4^+ concentration on the GTPase activity of parental strain (panel A) and kirromycin-resistant (panel B) EF-Tu alone (●), plus Phe-tRNA^{Phe} (△), or plus ribosomes (○). The reaction mixture contained, in 75 μL of 50 mM imidazolium acetate, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, and NH_4Cl as indicated, 12 pmol of EF-Tu·GDP from the different strains, 12 pmol of EF-Ts, 72 pmol of [γ-^{32}P]GTP (sp act. 2176 cpm/pmol), and, when indicated, 60 pmol of Phe-tRNA^{Phe} or 20 pmol of ribosomes. The reaction was carried out either for 40 min (●, △), or 10 min (○) at 37 °C. Blanks of EF-Ts with [γ-^{32}P]GTP ($^1/_5$ of the maximal activity) or with ribosomes and [γ-^{32}P]GTP ($^1/_4$ of the maximal activity) were subtracted.

EF-Tu·GDP-GTP Exchange Reaction. This assay was performed by adding either the radioactive [γ-^{32}P]GTP to the unlabeled EF-Tu·GDP complex or an excess of purified GTP (to avoid traces of GDP) to the EF-Tu·[^{3}H]GDP complex, formed by incubating stoichiometric amounts of nucleotide-free EF-Tu and [^{3}H]GDP. Determination of the radioactive nucleotide bound to EF-Tu was carried out by filtration of aliquots of the incubation mixture on nitrocellulose filters (Millipore or Sartorius), which were washed once by 3 mL of cold incubation buffer, dried, and counted in 5 mL of toluene-2,5-diphenyloxazole (5g/L).

The elongation factor Ts (EF-Ts), when present in this reaction, as well in the GTPase assay, was that obtained from *E. coli* B. No difference was detected with EF-Ts from *E. coli* D22 or D2216.

Assay for GTPase Activity. [γ-^{32}P]GTP hydrolysis was measured as liberation of inorganic phosphate, after addition of $HClO_4$ and extraction with isopropyl acetate in the presence of sodium molybdate (Sander et al., 1975).

Miscoding for Leucine. Miscoding was measured as the ratio between [^{3}H]leucine and [^{14}C]phenylalanine incorporated into acid-insoluble material in a poly(U)-dependent protein synthesizing system containing different concentrations of $MgCl_2$ (6–20 mM) and of postribosomal supernatant (0.6–3 μL) from D22 or D2216 *E. coli*. The incubation time was within the linear range of the incorporation kinetics.

Results

Absolute Requirement for NH_4^+ of Endogenous GTPase of EF-Tu$_{D2216}$: Effect of Ribosomes and aa-tRNA. Unlike wild-type EF-Tu, kirromycin-resistant EF-Tu$_{D2216}$ displays a GTPase activity in the absence of aminoacyl-tRNA and ribosomes (see preceding paper in this issue). Just as we have demonstrated for the kirromycin-induced GTPase activity in wild-type EF-Tu (Sander et al., 1979), this EF-Tu$_{D2216}$ GTPase activity is absolutely dependent on the presence of ammonium ions, with a plateau of maximum activity displayed at [NH_4^+] higher than 200 mM (Figure 1B; cf. the control wild-type EF-Tu in the upper panel of Figure 1A). In the presence of Phe-tRNA^{Phe}, the plateau of maximum activity is little modified, but maximum activity is reached at lower ammo-
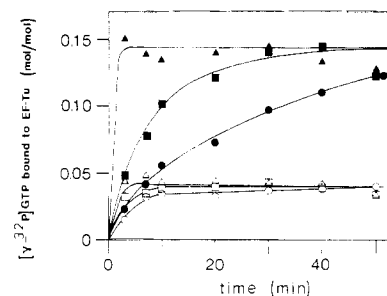

FIGURE 2: EF-Tu·GDP-[γ-^{32}P]GTP exchange at 0 °C. The reaction mixture contained, in 1 mL of 50 mM imidazolium acetate, pH 7.5, 10 mM $MgCl_2$, 60 mM NH_4Cl, and 1 mM dithiothreitol, 60 pmol of EF-Tu$_{D22}$·GDP (○, □, △) or EF-Tu$_{D2216}$·GDP (●, ■, ▲), 470 pmol of [γ-^{32}P]GTP (sp act. 12 258 cpm/pmol) without EF-Ts (○, ●) or with 10 (□, ■) or 100 (△, ▲) pmol of EF-Ts. The reaction was started by the addition of EF-Tu·GDP to the other components; at the given times, aliquots of 100 μL were withdrawn from the reaction mixture and the bound radioactivity was determined as described under Materials and Methods.

nium concentration (~100 mM). A similar effect is also seen in the kirromycin-induced system with wild-type EF-Tu (Sander et al., 1979). Addition of ribosomes to EF-Tu$_{D2216}$ causes a 5-fold stimulation of the maximum GTPase activity which shows a defined optimum at 100 mM NH_4^+, the same as that observed in both the kirromycin- and methanol-induced GTPase activities of wild-type EF-Tu in the presence of ribosomes (Ballesta & Vazquez, 1973; Sander et al., 1979). In the experiments reported in Figure 1, an equimolar quantity of EF-Ts was always added to EF-Tu to accelerate the recycling of EF-Tu·GTP from EF-Tu·GDP (see next section).

These results clearly indicate that the endogenous GTPase activity of mutant EF-Tu$_{D2216}$ discloses characteristics very similar to those observed with the GTPase activity of wild-type EF-Tu in the presence of kirromycin and partially also with those in the presence of methanol.

Kirromycin-Resistant EF-Tu$_{D2216}$ Has an Increased Affinity for GTP. We have used the competition between GDP and GTP for binding to EF-Tu as a probe to investigate the properties of the nucleotide binding site of the mutant and wild-type factor. The EF-Tu·GDP–GTP exchange rates were measured at 0 °C for both parental and mutant EF-Tu to reduce to a minimum the hydrolysis of GTP by the latter. The reaction was carried out in the presence and absence of wild-type EF-Ts. As illustrated in Figure 2, at equilibrium conditions, a higher amount of EF-Tu·GTP complex is obtained with mutant EF-Tu$_{D2216}$ than with EF-Tu$_{D22}$. This suggests that the affinity for GTP, which is normally 2 orders of magnitude lower than that for GDP (Miller & Weissbach, 1970; Arai et al., 1974; Fasano et al., 1978), is increased in the mutant EF-Tu$_{D2216}$. The presence of EF-Ts stimulates the rate, but not the extent, of the GTP exchange, confirming that EF-Ts does not affect the equilibrium of the GTP and GDP complexes with EF-Tu (Fasano et al., 1978). Moreover, it indicates that the stimulatory effect of EF-Ts on the dissociation of GDP from EF-Tu is not affected by the mutation. This has been confirmed by the EF-Tu·GDP–GDP exchange reaction, which is identical with respect either to rate or to extent, whether EF-Ts is present or not, for both mutant and wild-type EF-Tu (data not shown).

The dissociation rate of GTP from the EF-Tu·GTP complex is much slower for the mutant than for the parental EF-Tu (Figure 3) and is the only rate affected in mutant EF-Tu (Table I). The increased affinity of EF-Tu$_{D2216}$ for GTP, which accompanies the appearance of the kirromycin resistance, is therefore caused by the decreased rate of dissociation
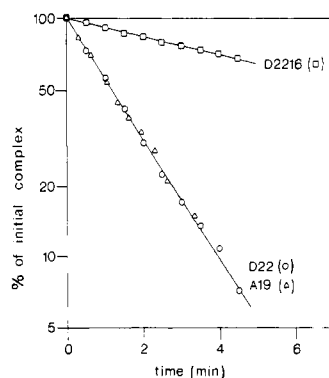
FIGURE 3: Rate of dissociation of GTP from kirromycin-resistant (□), wild-type (△), and parental strain (○) EF-Tu·GTP complex. The reaction mixture contained, in 1 mL of 50 mM imidazolium acetate, pH 7.5, 10 mM MgCl₂, 50 mM NH₄Cl, and 1 mM dithiothreitol, 30 pmol of EF-Tu from the different *E. coli* strains and 120 pmol of [γ-³²P]GTP (sp act. 6308 cpm/pmol). The proportion of EF-Tu complexes with GTP was 25% in the case of EF-Tu from strains of A19 (△) and D22 (○) and 62% in the case of EF-Tu from the kirromycin-resistant strain D2216 (□). The reaction was started by the addition of a 1000-fold excess of unlabeled nucleotide after 10 min of preincubation to allow formation of the EF-Tu·[γ-³²P]GTP] complexes. Incubation was at 0 °C; aliquots of 90 μL were filtered at the time indicated and the retained radioactivity was measured.
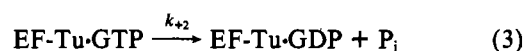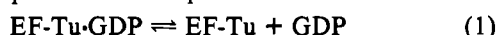
Table I: Affinity Constants at 0 °C for GTP and GDP of Kirromycin-Resistant and Parental Strain EF-Tu, Derived from Corresponding Apparent Rate Constants of Association and Dissociation[a]

	$10^{-4}k_{+1}'$ $(\text{s}^{-1}\,\text{M}^{-1})$	$10^{4}k_{-1}'$ (s^{-1})	$K' = k_{-1}'/k_{+1}'$ (nM)
EF-Tu$_{D22}$ (GTP)	2	96	480
EF-Tu$_{D2216}$ (GTP)	2	14.6	73
EF-Tu$_{D22}$ (GDP)	21	2.6	1.2
EF-Tu$_{D2216}$ (GDP)	21	2.6	1.2

[a] The association rates were measured in the presence of either 25 nM EF-Tu and 150 nM [γ-³²P]GTP (sp act. 4356 cpm/pmol) or 14 nM EF-Tu and 10 nM [³H]GDP (sp act. 6374 cpm/pmol), in 1-mL and 2-mL reaction mixtures, respectively, containing 50 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 50 mM NH₄Cl, and 1 mM dithiothreitol. The reaction was started by the addition of the labeled nucleotide, and aliquots of ¹/₁₀ the volume of the reaction mixture were then filtered on nitrocellulose filters at 5-s intervals. Dissociation rates were measured with either 30 nM EF-Tu·[γ-³²P]GTP (sp act. 6148 cpm/pmol) or 15 nM EF-Tu·[³H]GDP (sp act. 4500 cpm/pmol) in 1 mL of the same buffer as above. The reaction was started by the addition of a 1000-fold excess of unlabeled nucleotide, and the radioactive complexes were determined at appropriate intervals. Apparent association and dissociation rate constants were calculated as described under Materials and Methods.

between the factor and GTP. As with EF-Tu from wild-type strains, the dissociation rate of EF-Tu·GTP is perfectly in accord with a first-order kinetics, thus giving strong additional evidence that purified mutant EF-Tu represents a homogeneous population. Van de Klundert (1978) has shown that an independently obtained kirromycin-resistant EF-Tu has also a higher affinity for GTP than wild-type EF-Tu.

Mechanism of Stimulation by NH₄⁺ of Endogenous EF-Tu$_{D2216}$ GTPase Activity. EF-Tu-dependent turnover GTPase activity takes place via the steps

$$EF\text{-}Tu\cdot GDP \rightleftharpoons EF\text{-}Tu + GDP \qquad (1)$$

$$EF\text{-}Tu + GTP \rightleftharpoons EF\text{-}Tu\cdot GTP \qquad (2)$$

$$EF\text{-}Tu\cdot GTP \xrightarrow{k_{+2}} EF\text{-}Tu\cdot GDP + P_i \qquad (3)$$

In wild-type EF-Tu, the equilibria of reactions 1 and 2 are shifted toward the formation of EF-Tu·GDP (Miller &

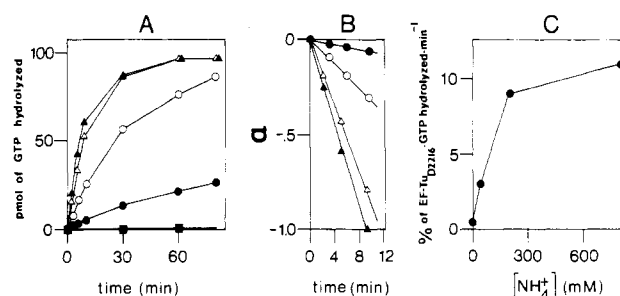FIGURE 4: Rate of hydrolysis of the EF-Tu$_{D2216}$·[γ-³²P]GTP complex. (Panel A) Time course of the reaction. The reaction mixture contained, in 500 μL of 50 mM imidazolium acetate, pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol, and NH₄Cl as indicated, 200 pmol of nucleotide-free EF-Tu$_{D2216}$. After addition of 100 pmol of [γ-³²P]GTP (sp act. 3663 cpm/pmol) and preincubation for 15 min at 0 °C to allow formation of the EF-Tu$_{D2216}$·[γ-³²P]GTP complex, the reaction was started by addition of NH₄Cl to the appropriate final concentration, together with a temperature shift to 37 °C. Aliquots of 60 μL were withdrawn at the time indicated and the ³²Pᵢ released was determined as described under Materials and Methods. (▲) 800 mM, (△) 200 mM, (○) 40 mM, and (●) 0 mM ammonium. (■) Blank of [γ-³²P]GTP alone. (Panel B) First-order plot of the EF-Tu$_{D2216}$·[γ-³²P]GTP hydrolysis. The rate of hydrolysis of the EF-Tu$_{D2216}$·[γ-³²P]GTP complex to EF-Tu$_{D2216}$·GDP and ³²Pᵢ, which takes place during the first 10 min of the reaction illustrated in (A), was plotted according to $a = -\ln\,([EF\text{-}Tu_{D2216}\cdot[\gamma\text{-}^{32}P]GTP]_{orig} - [^{32}P_i]_t)/[EF\text{-}Tu_{D2216}\cdot[\gamma\text{-}^{32}P]GTP]_{orig}$ vs. time, where EF-Tu$_{D2216}$·[γ-³²P]GTP$_{orig}$ is the concentration of the complex, formed as described in the legend to (A), at zero time. Since the nucleotide-free EF-Tu$_{D2216}$ was in 2-fold excess over the [γ-³²P]GTP, and at a concentration (400 nM) which is much higher than its affinity constant for GTP (73 nM, see Table I), [EF-Tu$_{D2216}$·[γ-³²P]GTP]$_{orig}$ is equivalent to the concentration of the [γ-³²P]GTP originally added. (Panel C) Apparent first-order rate constants for EF-Tu$_{D2216}$·GTP hydrolysis at different ammonium concentrations, estimated from (B).

Weissbach, 1970); furthermore, reaction 3 occurs only when appropriate effectors (e.g., aminoacyl-tRNA and ribosomes or kirromycin) are also present (Fasano et al., 1978).

The occurrence of a spontaneous GTPase activity with mutant EF-Tu (Figure 1) could be explained either by the increased formation of the EF-Tu·GTP complex though reaction 2 and/or by an increased rate of the hydrolytic step (reaction 3). Since GTPase activity is strongly dependent on NH₄⁺ (Figure 1), we decided to assess the influence of this cation on the individual steps of the turnover GTPase reaction with mutant EF-Tu.

We investigated steps 1 and 2 by making use of the EF-Tu·GDP–GTP exchange reaction performed at 0 °C to avoid the hydrolysis of GTP. The results, not illustrated, showed that at low [NH₄⁺], a condition in which there is no detectable GTPase activity, EF-Tu·GTP complex was formed to a greater extent than at higher [NH₄⁺]. Thus the increased affinity for GTP of the mutant EF-Tu cannot per se be the cause of the GTPase activity.

In order to investigate reaction 3, we prepared a defined amount of EF-Tu$_{D2216}$·[γ-³²P]GTP by incubating [γ-³²P]GTP with a 2-fold excess of nucleotide-free EF-Tu$_{D2216}$ at 0 °C. The rate of hydrolysis (Figure 4A), following a temperature shift to 37 °C, obeys first-order kinetics (Figure 4B) and thus allows evaluation of the apparent k_{+2}. In Figure 4C the values of k_{+2} obtained at different [NH₄⁺] are shown: not only does increasing [NH₄⁺] strongly raise the rate of reaction 3, but also the presence of this cation appears to be essential for the hydrolytic step. The finding that the rate of GTP hydrolysis of the EF-Tu·[γ-³²P]GTP complex followed first-order kinetics ruled out the possibility that a contaminant, present in trace amounts, could have been responsible for the endogenous GTPase activity.

Table II: Influence of Kirromycin and EF-Ts on the Dissociation of GTP and GDP from Parental and Mutant EF-Tu[a]

	parental strain (min)	mutant (min)
EF-Tu·GTP	1.2	7.9
EF-Tu·GTP + kirromycin (1 μM)	52	8
EF-Tu·GTP + kirromycin (100 μM)	52	30
EF-Tu·GTP + EF-Ts (50 nM)	0.1	0.8
EF-Tu·GTP + kirromycin (1 μM) + EF-Ts (50 nM)	52	0.8
EF-Tu·GTP + kirromycin (100 μM) + EF-Ts (50 nM)	52	25
EF-Tu·GDP	44	44
EF-Tu·GDP + kirromycin (1 μM)	31	44
EF-Tu·GDP + kirromycin (100 μM)	15	44
EF-Tu·GDP + EF-Ts (20 nM)	0.9	0.9
EF-Tu·GDP + kirromycin (1 μM) + EF-Ts (20 nM)	9.5	0.9
EF-Tu·GDP + kirromycin (100 μM) + EF-Ts (20 nM)	15	0.9

[a] Apparent dissociation rate constants of the EF-Tu·nucleotide complexes are expressed as half-lives. The reaction mixture contained, in 2 mL of 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 50 mM NH_4Cl, and 1 mM dithiothreitol, either 30 nM EF-Tu·[γ-^{32}P]GTP complex (sp act. 5800 cpm/pmol) or 15 nM EF-Tu·[^{3}H]GDP complex (sp act. 4500 cpm/pmol), and other components as indicated. The reaction was started by the addition of EF-Ts, when this component was required, and a 1000-fold excess of unlabeled nucleotide. Incubation was at 0 °C, aliquots of 200 μL were withdrawn from the reaction mixture at appropriate intervals, ranging from 5 s to 8 min, and the radioactive complexes were determined by the nitrocellulose filter procedure.

Additional evidence that the GTPase is associated with the EF-Tu molecule came from the observation that, after addition of increasing amounts of GDP to nucleotide-free EF-Tu, the initial rate of [γ-^{32}P]GTP hydrolysis was greatly reduced: 150, 500, or 1000 pmol of GDP added to a reaction mixture of 250 μL, containing 135 pmol of EF-Tu and 450 pmol of [γ-^{32}P]GTP, caused an inhibition of 65, 92, and 97%, respectively. This was expected from the negative effect of GDP on the formation of the EF-Tu·[γ-^{32}P]GTP complex. In fact, in the presence of GDP, formation of EF-Tu·GDP is favored compared to that of EF-Tu·GTP (Miller & Weissbach, 1970; Fasano et al., 1978). Our results on the inhibition by GDP of the spontaneous GTPase activity of EF-Tu$_{D2216}$ imply a higher affinity of EF-Tu for GDP rather than for GTP, a characteristic which is shared by wild-type and mutant EF-Tu (Table I).

Interaction of Mutant EF-Tu$_{D2216}$ with Aminoacyl-tRNA and Kirromycin. No modification of the stabilizing effect of aminoacyl-tRNA on the EF-Tu·GTP complex, observed with wild-type EF-Tu (Fasano et al., 1978), is found with the kirromycin-resistant EF-Tu$_{D2216}$. The same is true for the stimulation by EF-Ts of the dissociation rate of the EF-Tu$_{D2216}$·GDP complex, as already suggested from the effect of EF-Ts on the EF-Tu·GDP–GTP exchange reaction described in a previous section.

An interesting result was obtained when we investigated the influence of kirromycin on the interaction between EF-Tu$_{D2216}$ and GTP or GDP: the antibiotic was still able to inhibit the rate of dissociation of GTP from mutant EF-Tu·GTP complex, even though a much higher concentration was required as compared with wild-type EF-Tu·GTP (Table II). By contrast, the same concentration of kirromycin (100 μM) was unable to modify either the rate of dissociation of GDP from mutant EF-Tu$_{D2216}$·GDP or the rate of association of GDP and GTP to nucleotide-free EF-Tu$_{D2216}$ (not shown). These findings confirm the results of Fasano et al. (1978) that kirromycin
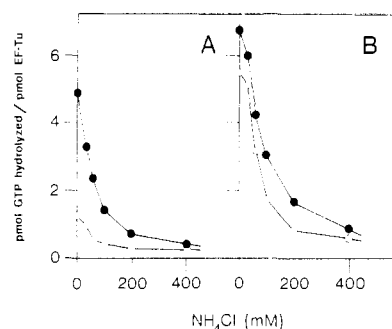

FIGURE 5: Effect of [NH_4^+] on poly(U)-dependent (●) and poly-(U)-independent (○) GTPase activity of parental strain (panel A) and kirromycin-resistant (panel B) EF-Tu. The reaction mixture contained, in 75 μL of 50 mM imidazolium acetate, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, and NH_4Cl as indicated, 12 pmol of EF-Tu·GDP from the different strains, 12 pmol of EF-Ts, 310 pmol of [γ-^{32}P]GTP (sp act. 2122 cpm/pmol), 20 pmol of ribosomes, 60 pmol of Phe-tRNA^{Phe}, and (●) 4 μg of poly(U). Incubation was carried out for 4 min at 37 °C.

interacts preferentially with EF-Tu·GTP. That the mutant EF-Tu is still somewhat sensitive to kirromycin is also shown by the observation of Fischer et al. (1977) that GTPase activity can be enhanced in EF-Tu$_{D2216}$ by high concentrations of the antibiotic.

It has been shown that the binding of kirromycin to the wild-type EF-Tu·guanine nucleotide complex does not allow the subsequent binding of EF-Ts (Chinali et al., 1977; Fasano et al., 1978). Therefore, it is reasonable to assume that binding of the antibiotic to the mutant EF-Tu$_{D2216}$ would prevent any stimulatory effect by EF-Ts on the dissociation rate of EF-Tu$_{D2216}$·GDP. From Table II it appears that kirromycin, even at very high concentrations, is completely unable to antagonize the stimulatory effect of EF-Ts on the dissociation of GDP from mutant EF-Tu. The same holds true when low concentrations of kirromycin are used to inhibit the effect of EF-Ts on the dissociation of GTP from EF-Tu$_{D2216}$·GTP, a 1 μM concentration of the antibiotic being fully active in prohibiting the action of EF-Ts on wild-type EF-Tu but completely ineffective with mutant EF-Tu$_{D2216}$. Higher concentrations of kirromycin, however, did exert some effect when EF-Tu$_{D2216}$·GTP was used. These results confirm that on the mutant EF-Tu$_{D2216}$ there is a binding site with a reduced affinity for the antibiotic, becoming evident only when the factor is complexed with GTP.

Properties of mRNA-Dependent GTPase Activity of EF-Tu$_{D2216}$. The GTPase activity of EF-Tu, which normally accompanies the interaction between the ternary complex EF-Tu·GTP·aa-tRNA and the mRNA·complex, can be uncoupled from polypeptide synthesis by omitting elongation factor G (Gordon, 1969). When tested in this system as a function of [NH_4^+], the mutant EF-Tu$_{D2216}$ appears to be more active than the parental EF-Tu$_{D22}$ (Figure 5) which is in turn identical with wild-type EF-Tu from *E. coli* strains B or A19 (not shown).

At the level of Mg^{2+} used in this assay (10 mM), the GTPase activity of parental EF-Tu$_{D22}$ is largely dependent on the presence of mRNA (Sander, 1977), such that when tested in the absence of poly(U) (Figure 5A), little activity is recorded. This appears to be in marked contrast to mutant EF-Tu$_{D2216}$, where in the absence of poly(U) (Figure 5B) and without added monovalent cations more than 80% of its GTPase activity is maintained.

Since increasing the [Mg^{2+}] to 25 mM has been shown to relieve the GTPase activity of wild-type EF-Tu from its mRNA requirement (Sander, 1977), the effect of the presence
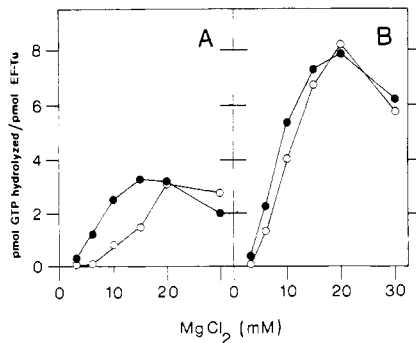
FIGURE 6: Effect of $[Mg^{2+}]$ on poly(U)-dependent (●) and poly-(U)-independent (O) GTPase activity of parental strain EF-Tu (panel A) and kirromycin-resistant EF-Tu (panel B). The reaction mixture contained, in 75 μL of 50 mM imidazolium acetate, pH 7.5, 30 mM NH_4Cl, 1 mM dithiothreitol, and $MgCl_2$ as indicated, 6 pmol of EF-Tu·GDP from the different strains, 6 pmol of EF-Ts, 330 pmol of $[\gamma-^{32}P]GTP$ (sp act. 2122 cpm/pmol, 10 pmol of ribosomes, 45 pmol of Phe-tRNA^{Phe}, and (●) 4 μg of poly(U). Incubation was for 5 min at 37 °C.

of poly(U) on the EF-Tu-dependent GTPase activity was also tested as a function of $[Mg^{2+}]$ (Figure 6). At 6 mM $MgCl_2$, a concentration at which wild-type or parental EF-Tu is almost 100% dependent on poly(U) (panel A), EF-Tu$_{D2216}$ exhibits 60% of its mRNA-stimulated activity already in the absence of poly(U) (panel B).

We have also tested the GTPase activity of mutant EF-Tu$_{D2216}$ in the presence of an excess of ribosomes and 20% methanol. This system allows the expression of the EF-Tu-dependent GTPase activity seen in the absence of amino-acyl-tRNA and mRNA (Ballesta & Vazquez, 1973). The EF-Tu$_{D2216}$ was about twice as active as the wild-type EF-Tu, the former hydrolyzing ~3 pmol of GTP/(min pmol of EF-Tu) and the latter only 1.5 pmol.

Miscoding Properties of Mutant and Parental Strain EF-Tu. We have compared mutant and parental strain EF-Tu in the poly(U)-directed poly(phenylalanine/leucine) synthesis at different Mg^{2+} concentrations, with the postribosomal supernatant as a source of EF-Tu. While increasing $[Mg^{2+}]$ did induce a higher ratio of leucine/phenylalanine incorporation into acid-insoluble material in accord with the results of Davies et al. (1964), no difference was detectable when EF-Tu from either D22 or D2216 was used (these results are not illustrated).

Discussion

In spite of the apparent functional similarity of the parental and mutant EF-Tu in the poly(U)-directed poly(phenylalanine) synthesis system and in the stimulation of enzymatic binding of aminoacyl-tRNA to the ribosome (see preceding article in this issue), the mutation in the EF-Tu from strain D2216 does influence the factor-mediated GTPase activity. In fact, in the absence of any of the normal allosteric effectors, a GTPase activity can be measured in the mutant factor. Moreover, in the EF-Tu-dependent GTPase system containing also amin-oacyl-tRNA, ribosomes, mRNA, and EF-Ts, the rate of GTP hydrolysis in the presence of EF-Tu$_{D2216}$ can be as much as double that in the presence of parental EF-Tu. This GTPase, in the case of the mutant, no longer requires mRNA under conditions where for wild-type EF-Tu there is an absolute requirement for this effector.

The striking similarity between the endogenous GTPase activity of the kirromycin-resistant EF-Tu$_{D2216}$ and that induced by kirromycin in wild-type EF-Tu, particularly in regard to the influence of ribosomes, aminoacyl-tRNA, and ammo-

nium ions, suggests that there is a common region on the EF-Tu molecule regulating the selective expression of this catalytic center. A perturbation of this region, either by binding of the antibiotic kirromycin in the wild-type EF-Tu or by structural modification due to a changed peptide map in the mutant EF-Tu (Fischer et al., 1977), allows the expression of the GTPase activity in the absence of any other effector.

A detailed analysis of the interactions of the mutant EF-Tu$_{D2216}$ with various effectors showed that the relationships with aminoacyl-tRNA, EF-Ts, and GDP are essentially unchanged. The mutation caused a large decrease in the affinity of the elongation factor for kirromycin and also increased the affinity of EF-Tu$_{D2216}$ for GTP via a lowering of the EF-Tu·GTP dissociation rate. We could show that this increased affinity for GTP cannot per se be the cause of the endogenous GTPase activity, though it clearly stimulates the recycling of GDP by GTP; the increased affinity for the substrate and the more catalytically active conformation of the mutant EF-Tu$_{D2216}$ together favor the turnover GTPase activity.

It should be noted that, in experiments to be published elsewhere, we observed that spontaneous hydrolysis of wild-type EF-Tu·GTP to EF-Tu·GDP and P$_i$ in the absence of any effector may also occur. The reason that this is not manifest as a turnover GTPase activity starting with EF-Tu·GDP is that the affinity for GTP of wild-type EF-Tu is 1 order of magnitude lower than in the case of mutant EF-Tu$_{D2216}$, thus preventing a replacement of GDP by GTP in the nucleotide binding site on the factor.

The GTPase activity of mutant EF-Tu$_{D2216}$ is strongly stimulated by ribosomes and Phe-tRNA^{Phe} at low Mg^{2+} concentrations (6 mM) in the absence of poly(U) (Figure 6B), whereas the latter component is strictly required for the expression of the GTPase activity of wild-type EF-Tu (Figure 6A). This suggests that the mutant factor does not obey as effectively as wild-type EF-Tu the constraints imposed by the codon–anticodon interaction.

It has been suggested that GTP hydrolysis is involved in the recognition process (Thompson & Stone, 1977); however, the altered expression of the GTPase activity in the mutant EF-Tu does not appear to influence the fidelity of translation.

Kirromycin has been shown to bind preferentially to EF-Tu·GTP, rather than to EF-Tu·GDP (Fasano et al., 1978; Douglass & Blumenthal, 1979). The observation that kirromycin, at high concentrations, can still bind to EF-Tu$_{D2216}$·GTP, whereas the same does not occur with EF-Tu$_{D2216}$·GDP, confirms that the EF-Tu conformation induced by GTP shows a higher affinity for the antibiotic and emphasizes the role of the guanine nucleotides as allosteric effectors of the EF-Tu functions.

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Dynamic Properties of the Lipid–Water Interface of Model Membranes As Revealed by Lifetime-Resolved Fluorescence Emission Spectra[†]

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ABSTRACT: We examined the dynamic properties of the lipid–water interface region of model membranes, on the nanosecond time scale, by using the fluorescent probe 2-*p*-toluidinylnaphthalene-6-sulfonic acid (TNS). In particular, we examined the steady-state emission spectra of TNS as its average lifetime was decreased by oxygen quenching. Under these quenching conditions the centers of gravity (ν_{cg}) of the emission spectra shift to shorter wavelengths. The lifetime dependence of these shifts reveals the time dependence of membrane relaxation around the excited-state dipole moment of TNS. The lipids investigated include dioleoyl-, dimyristoyl-, and dipalmitoyl-L-α-phosphatidylcholines, bilayers containing cholesterol, and an ether analogue of dipalmitoyl-L-α-phosphatidylcholine. For these lipids, the spectral relaxation times and the temperature dependence of the relaxations are similar in magnitude. Most relaxation times fall in the range of 0.6–6 ns, and except for the ether analogue, the activation energies for spectral relaxation are 10 ± 2 kcal/mol. The average energy loss during spectral relaxation was 1000 cm⁻¹. However, for the saturated phosphatidylcholines at temperatures below their transition temperatures, smaller relaxation losses were observed (~600 cm⁻¹). We attribute these smaller losses to ordering of the polar head groups around the ground-state dipole moment of TNS. Overall, these results indicate that the dynamic properties of the lipid–water interface region are similar among the phosphatidylcholines and depend only slightly on the chemical composition and phase state of the acyl side chains.

The functional properties of cell membranes appear to be dependent upon the dynamic behavior of the phospholipid molecules (Chapman, 1976; Melchior & Steim, 1976; Singer & Nicolson, 1972). For example, the permeabilities of model membrane to ions and nonelectrolytes and the activities of membrane-bound enzymes are dependent upon the chemical composition, temperature, and phase state of the lipid bilayers. Recently, much attention has been directed toward elucidating the dynamic properties of the acyl side chain region of lipid bilayers as revealed by their apparent microviscosities (Shinitzky et al., 1971; Cogen et al., 1973; Lentz et al., 1976; Lakowicz et al., 1979a,b). In the current investigation we characterize further the dynamic properties of model membranes but now with emphasis on the lipid–water interfacial region. In particular, we investigated the ability of this region to undergo relaxation around the excited-state dipole moment of 2-*p*-toluidinylnaphthalene-6-sulfonic acid (TNS).[1] This probe and other similarly charged fluorophores are known to bind to the polar head-group region of membranes (Jendrasiak & Estep, 1977; Lesslauer et al., 1971, 1972; Radda, 1971). The dipolar relaxation rate of the membrane is revealed by the rate at which the fluorescence emission of TNS shifts to longer wavelengths following excitation.

Generally, the time evolution of excited states is determined by fluorescence emission spectra obtained at nanosecond intervals following pulsed excitation, as has been described for fluorophores in solvents (Ware et al., 1968; Mazurenko, 1973) and in proteins and membranes (Brand & Gohlke, 1971; DeToma et al., 1976; Gafni et al., 1977). These time-dependent spectral shifts result from reorientation of the molecules surrounding the fluorophore around the changed (generally increased) dipole moment of the excited state. Under

[1] Abbreviations used: TNS, 2-*p*-toluidinylnaphthalene-6-sulfonic acid; DOPC, DMPC, and DPPC, dioleoyl-, dimyristoyl-, and dipalmitoyl-L-α-phosphatidylcholine, respectively; DPPC-E, L-α-phosphatidylcholyl dipalmitoyl ether; Chol, cholesterol; Tris, tris(hydroxymethyl)aminomethane; P_{O_2}, psi of O_2 pressure used.