# Characterization of a Kirromycin-Resistant Elongation Factor Tu from *Escherichia coli*†

Richard Ivell,‡ Ottavio Fasano,§ Jean-Bernard Crechet, and Andrea Parmeggiani*

ABSTRACT: The *Escherichia coli* strain D2216 contains a kirromycin-resistant elongation factor Tu [EF-$Tu_{D2216}$; Fischer, E., Wolf, H., Hantke, K., & Parmeggiani, A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4341–4345]. This strain grows much more slowly than wild-type *E. coli* strains and contains less than half the amount of EF-Tu. On isoelectric focusing, the whole cell lysate of strain D2216 as well as pure, crystalline EF-$Tu_{D2216}$ comprises only a single species indistinguishable from wild-type EF-Tu. In poly(uridylic acid)- [poly(U)] directed poly(phenylalanine) synthesis, enzymatic binding of aminoacyl transfer ribonucleic acid to the ribosome, and susceptibility to trypsin digestion, EF-$Tu_{D2216}$ behaves similarly to the EF-Tu from wild-type strains. Kirromycin, which increases the sensitivity to trypsinization of wild-type EF-Tu, has no effect on mutant EF-Tu. In poly(U)-directed poly(phenylalanine) synthesis, partially trypsinized EF-$Tu_{D2216}$ displays a 7-fold reduction of its kirromycin resistance as compared to the intact EF-$Tu_{D2216}$. This is ~300 times less sensitive to the antibiotic than wild-type EF-Tu. The EF-$Tu_{D2216}$, purified and crystallized, exhibits a guanosine 5′-triphosphatase activity in the absence of any other physiological effector or kirromycin. This activity is not a contaminant, since it can be selectively stimulated by ribosomes and is inactivated by temperature exactly in the same way as the guanosine 5′-diphosphate binding activity of EF-$Tu_{D2216}$. We conclude that, as consequence of the mutation, the catalytic center of EF-$Tu_{D2216}$-dependent guanosine 5′-triphosphate hydrolysis undergoes spontaneous activation.

The inhibition of peptide bond formation by the antibiotic kirromycin takes place via elongation factor Tu (EF-Tu),[1] which is blocked on the ribosome after the binding of aminoacyl-tRNA and the associated GTP hydrolysis (Wolf et al., 1974; Chinali et al., 1977; Wolf et al., 1977; Parmeggiani & Sander, 1980). Kirromycin globally affects the functions of EF-Tu by modifying not only the affinity of the factor for the ribosome, but also its interactions with GTP, GDP, aminoacyl-tRNA, and elongation factor Ts (EF-Ts) (Fasano et al., 1978a). The GTPase activity of EF-Tu induced by the antibiotic is uncoupled from the presence of the ribosome and aminoacyl-tRNA, though these two components, individually or in combination, show a stimulatory effect (Chinali et al., 1977; Sander et al., 1979). Recently, *Escherichia coli* mutants carrying a kirromycin-resistant EF-Tu have been isolated (Fischer et al., 1977; Van de Klundert et al., 1977). This was of considerable interest because of the existence in wild-type strains of two genes coding for EF-Tu (Jaskunas et al., 1975; Furano, 1978) and the phenotypic dominance of kirromycin sensitivity over kirromycin resistance due to the mode of action of the antibiotic (Parmeggiani et al., 1976; Wolf et al., 1977; Van de Klundert et al., 1977; Fischer et al., 1977).

In view of the functional importance of the mutation, we have carried out a comparative study of some of the properties of mutant ET-Tu from *E. coli* D2216 and those of the EF-Tu from the parental strain D22, as well as from more generally used wild-type strains. Analysis of both cell lysate and pure crystalline factor points to the homogeneity of the mutant EF-$Tu_{D2216}$. Most prominent amongst the modifications accompanying kirromycin resistance is a greater predisposition by the mutant EF-$Tu_{D2216}$ toward a catalytic conformation, such that the factor expresses a GTPase activity in the absence of any effector.

## Materials and Methods

All biological components, materials, and methods not mentioned in this section were as already reported (Sander et al., 1975; Chinali et al., 1977; Fischer et al., 1977). Kirromycin was a gift from Dr. H. Wolf, Tübingen, and was kept as a 10 mM mother solution in 100% ethanol at −30 °C.

Electrophoretically homogeneous crystalline EF-Tu from *E. coli* strains B and D22 (Normark et al., 1969; Monner et al., 1971) was purified by following the method of Chinali et al. (1977). With kirromycin-resistant EF-Tu from *E. coli* D2216, the purification step on DEAE-Sephadex A-50 was carried out twice (Fischer et al., 1977). Long-chain poly(U) and ribosomes containing more than 85% "tight couples" (Noll & Noll, 1976), were prepared as reported (Chinali & Parmeggiani, 1973; Sander et al., 1975). *E. coli* MRE600 was obtained from the Microbiological Research Establishement, England; other *E. coli* strains were grown as described (Chinali et al., 1977; Fischer et al., 1977).

*Cell Preparation for Determining the in Vivo Content of EF-Tu.* All measurements of the in vivo quantity and distribution of elongation factor Tu were made with freshly grown cultures of *E. coli*. The strains used were A19, $BT2^r$, MRE600, the kirromycin-resistant mutant D2216, and its

† From the Laboratoire de Biochimie (Laboratoire Associé No. 240 du Centre National de la Recherche Scientifique), Ecole Polytechnique, 91128 Palaiseau Cedex, France. *Received November 26, 1979; revised manuscript received August 13, 1980.* This work was supported by grants of the Délégation Générale à la Recherche Scientifique et Technique (No. 76.7.1186 and 78.7.1097) and by the Commissariat à l'Energie Atomique.

‡ Present address: Institut für Physiologische Chemie, Abt. Zellbiochemie, Universität Hamburg, Martinistr. 52, 2000 Hamburg 20.40, Federal Republic of Germany.

§ Recipient of a short-term E.M.B.O. fellowship. Present address: Cattedra di Chimica, II^a Facoltà di Medicina e Chirurgia, Università degli Studi, I-80131 Napoli, Italy.

[1] Abbreviations used: EF-Tu, elongation factor Tu; EF-Ts, elongation factor Ts; EF-G, elongation factor G; EF-$Tu_B$, EF-$Tu_{D22}$, and EF-$Tu_{D2216}$, elongation factor Tu from *E. coli* B, D22, and D2216, respectively; $tRNA^{Phe}$, phenylalanine-accepting transfer ribonucleic acid; S30, supernatant of the cell extract obtained after two centrifugations at 30000*g* for 4 h; S100, supernatant of the cell extract obtained from the S30, after centrifugation at 100000*g* for 5 h; PW, pellet wash; $\theta_{1/2}$, temperature at which 50% of EF-Tu·GDP is inactivated after 8 min of incubation; $NaDodSO_4$, sodium dodecyl sulfate; GTP, guanosine 5′-triphosphate; GTPase, guanosine 5′-triphosphatase; enzyme·GTPase (EC 3.6.1.–); poly(U), poly(uridylic acid); DEAE, diethylaminoethyl; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; TPCK, tosylphenylalanine chloromethyl ketone; mRNA, messenger ribonucleic acid.

0006-2960/81/0420-1355$01.25/0 

immediate parental strain D22. Cultures were grown in tryptone–NaCl media [13 g of tryptone (Oxoid) and 7 g of NaCl/L of deionized water] at 37 °C from stock innocula stored in 50% glycerol at −20 °C. Except where indicated, all cells were harvested in mid-log phase by pouring onto crushed frozen buffer containing 3 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.8, and 60 mM $NH_4Cl$ at −75 °C (500 g of frozen buffer/L of culture). After centrifugation, the pellets were gathered, washed once in the same buffer, and then resuspended by using a Potter-Elvehjem homogenizer in either a high-salt sonication buffer (750 mM $NH_4Cl$, 3 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.5, 25 mg/mL phenylmethanesulfonyl fluoride, 0.5 mM dithiothreitol, and 10 μM GDP) or a low-salt sonication buffer (as above but 750 mM $NH_4Cl$ is replaced by 150 mM KCl) at a concentration of 1 g of cells/10 mL of buffer. This suspension was sonicated for 3 × 30 s (MSE Ultrasonic Disintegrator, 150 W), with at least 1-min intervals in ice between sonication bouts. This treatment was found to result in maximum total protein and maximum EF-Tu yields when compared with alternative methods of cell lysis (data not shown). The cell lysate was then centrifuged at 30000*g* for 4 h at 2 °C to produce a 30000*g* supernatant (S30) and pellet. When the high-salt sonication buffer was used, the S30 was subsequently dialyzed overnight against the low-salt sonication buffer. The pellet was resuspended by using the Potter-Elvehjem homogenizer in low-salt sonication buffer plus 0.1% sodium deoxycholate and recentrifuged at 30000*g* for 4 h at 2 °C to give the pellet wash (PW). Where indicated, the S30 supernatant was recentrifuged at 100000*g* for 5 h at 2 °C to yield an S100 supernatant.

*Determination of EF-Tu Content.* Because EF-Tu has a very high affinity for GDP ($K'$ = 1 nM at 0 °C; Fasano et l., 1978a) and no other *E. coli* proteins are considered to bind GDP to a significant extent if the nucleotide is present in low concentration (Miller & Weissbach, 1977), the EF-Tu·GDP–[$^3$H]GDP exchange reaction is suitable for estimating the quantity of EF-Tu present in the cell extracts. The retention of the EF-Tu·[$^3$H]GDP on nitrocellulose filters (Sartorius SM11306) was measured after incubation for 5 min at 30 °C (Fasano et al., 1978a). To ensure a rapid completion of the reaction in the different cellular extracts, we added exogenous EF-Ts isolated from wild-type strains. EF-Ts from mutant strain also stimulated the EF-Tu·GDP–GDP exchange reaction. The total EF-Tu content was determined in several dilutions of the extract after sonication from the slope of straight-line plots of protein content vs. [$^3$H]GDP bound. External calibration using electrophoretically pure crystalline EF-Tu in various concentrations was also used as reference. EF-Tu content was also determined via poly(U)-directed poly(phenylalanine) synthesis (Wolf et al., 1974) carried out in conditions limited only by the concentration of EF-Tu.

*Isoelectric Focusing of Lysates and Pure Enzymes.* Isoelectric focusing of cell lysates and pure enzymes was carried out by following essentially the procedure of O'Farrell (1975). Lysates were prepared by sonication of freshly harvested and washed cells. The first-dimension gels (electrofocusing) were run for 14 h at 400 V followed by 3 h at 500 V. Where necessary, these first-dimension gels were then stained in 0.1% Coomassie Blue in 50% trichloroacetic acid for 15 min and then destained in successive changes of 15% acetic acid–10% ethanol. For the second electrophoresis dimension, the running gels were of 7.5% acrylamide–0.1% $NaDodSO_4$ (Pedersen et al., 1976b). These gels were developed at 20 mA constant current for 5–6 h, stained for 3 h in 0.125% Coomassie Blue–50% ethanol–5% acetic acid, and destained as described above. Stained first dimensions were scanned by using an ISCO gel scanner fitted with 620–nm filters.

*Trypsinization of EF-Tu.* Limited trypsinization of EF-Tu into 36K and 5K fragments (Arai et al., 1980; Wittinghofer et al., 1980) was carried out essentially as described by Arai et al. (1976) and Jacobson & Rosenbusch (1976). The reaction mixture (containing in 100 μL 0.7 nmol of EF-Tu, 50 mM Tris-HCl, pH 7.5, 7 mM $MgCl_2$, 60 mM $NH_4Cl$, and 1 mM dithiothreitol) was digested with 0.5 μg of trypsin (TPCK-treated, Merck) plus or minus 50 μM kirromycin, at 0 °C. At various time intervals, to aliquots of the solution was added an excess of soybean inhibitor (Boehringer). The EF-Tu, denatured in $NaDodSO_4$, was then applied to a slab gel formed with a 10–20% gradient of acrylamide, 0.2% bis-(acrylamide), and 1.5% $NaDodSO_4$, and electrophoresis was carried out in Tris-HCl, pH 8.8, at 20 °C for 5–6 h at a constant voltage of 100 V. The gels were stained and destained as described in the previous section for the second dimension of the O'Farrell procedure and dried under vacuum over a boiling water bath. The time for 50% trypsinization was visually estimated as the time at which the 43K native protein and its corresponding 36K fragment were of similar intensity.

*Determination of Thermal Inactivation Curves for Parental and Mutant EF-Tu.* Incubation mixtures were prepared, containing in 250 μL 50 mM imidazolium acetate, pH 7.5, 10 mM $MgCl_2$, 140 mM $NH_4Cl$, and 1 mM dithiothreitol with or without 50 μM kirromycin. To these were added 60 pmol of EF-$Tu_{D2216}$·[$^3$H]GDP complex, made by mixing 60 pmol of the appropriate EF-Tu with 250 pmol of [$^3$H]GDP (sp act. 1085 cpm/pmol). Each sample was incubated for 8 min at the temperatures indicated and then kept at 0 °C for 1 h. The EF-Tu activity was then determined for 50-μL aliquots by measuring the EF-Tu·[$^3$H]GDP complex retained on nitrocellulose filters (Sartorius SM 11308), after washing twice with 3 mL of cold (0 °C) buffer (10 mM $MgCl_2$, 50 mM imidazolium acetate, pH 7.5, and 140 mM $NH_4Cl$).

Alternatively, the activity of the mutant EF-$Tu_{D2216}$, incubated at different temperatures in the absence of kirromycin, was assessed by its unique ability to catalyze the hydrolysis of GTP in the presence or absence of ribosomes, without any other effector (see later). Fifty microliters of the incubation mixture was incubated together with 80 pmol of [$\gamma$-$^{32}$P]GTP (sp act. 2836 cpm/pmol) for 6 min at 30 °C in the presence of ribosomes and for 30 min in their absence. The final concentrations of $NH_4^+$ ion were adjusted to 200 mM in the absence of ribosomes or to 100 mM in their presence, i.e., to the optimal concentrations for these activities (see accompanying article). GTPase activity was assayed by the release of $^{32}P_i$ as previously described (Sander et al., 1975).

*Other Assays.* Poly(U)-directed poly(phenylalanine) synthesis, GTPase activity, EF-Tu-dependent binding of PhetRNA to the mRNA·ribosome were determined by the described procedures (Wolf et al., 1974; Sander et al., 1975; Chinali et al., 1977; Fischer et al., 1977). Protein concentration was determined by the method of Lowry et al. (1951), using bovine serum albumin as standard.

## Results

*Growth Rates and EF-Tu Content of Various E. coli Strains.* Recent studies have shown that of the two genes coding for EF-Tu in *E. coli*, *tufA* is expressed ~3 times more efficiently than *tufB* (Pedersen et al., 1976a,b); moreover, the intracellular quantity of EF-Tu has been linked to growth conditions (Pedersen et al., 1978). It was therefore of interest to examine whether expression of a kirromycin-resistant EF-Tu was accompanied by a modification of the EF-Tu content in

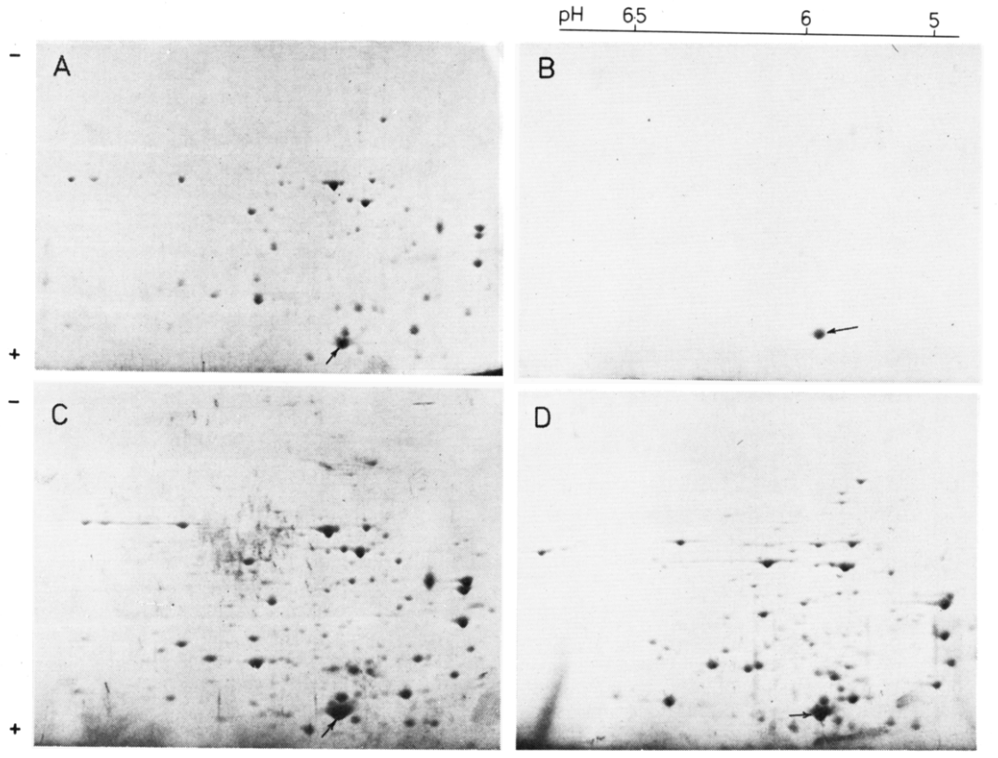


FIGURE 1: Bidimensional isoelectric focusing–electrophoresis of cell lysates or pure EF-Tu (see Materials and Methods). (A) D2216 lysate (~25 μg of protein); (B) pure crystalline wild-type EF-Tu (1 μg); (C) D2216 lysate (~40 μg of protein) plus pure crystalline wild-type EF-Tu (1 μg); (D) MRE600 lysate (~40 μg of protein). Arrow indicates EF-Tu.

the cell of our mutant D2216.

Under the chosen conditions, the wild-type strains all doubled approximately every 35 min. The parental strain D22, an *E. coli* K12 strain with an altered membrane (Normark et al., 1969; Monner et al., 1971) permeable to kirromycin (Fischer et al., 1977), is considerably slower, doubling in ~80 min. The kirromycin-resistant mutant is slower still, doubling approximately every 170 min. This may reflect not only the known mutation of EF-Tu, but also other mutations incurred by the treatment with nitrogen mustard (Fischer et al., 1977).

Table I shows the relative content of EF-Tu in the different strains. The total quantities of EF-Tu in the various strains were estimated for the combined 30000*g* supernatant (S30) and pellet wash (PW). From our results the EF-Tu content in wild-type strains represents ~9% of the total extractable protein. By contrast, both mutant D2216 and, to a lesser extent, parental D22 show reduced EF-Tu contents, possibly as a consequence of the reduced growth rate (Pedersen et al., 1978).

When the quantity of GDP-binding protein (assumed to be EF-Tu) was measured in the crude lysate, as well as in the S30, PW, and S100 extracts, it was found that nearly 90% of the total EF-Tu measured in the lysate was recovered from the S30 supernatant and pellet wash extract. With reference to the total cell protein, we determined an EF-Tu content in wild-type strains of ~5%, a value similar to that reported by other authors (4–5%, Furano, 1975; Jacobson & Rosenbusch, 1976; Herendeen et al., 1979).

A large portion of EF-Tu (~30%) was found to be associated with the pellet wash fraction, irrespective of the strain used. Whether this was all associated with the membrane via ribosomes remains to be investigated.

As a check on the identity of the GDP-binding protein in cell extracts, S30 supernatants from strains D2216, D22, and MRE600 were run on similar DEAE-Sephadex A-50 columns eluted with a linear gradient of 150–300 mM KCl, in the presence of 10 μM GDP. All the GDP-binding protein contained in the S30 supernatant was recovered as a single peak at ~200 mM KCl in all three strains and corresponded quantitatively to EF-Tu when measured by the EF-Tu limited peptidization assay (not illustrated). In all cases tested, this assay gave the same results as with the [$^3$H]GDP-binding method.

Table I: Quantities of EF-Tu and Total Extractable Protein in Various Strains

| | D2216 | D22 | MRE600, BT2$^r$, and A19 |
|---|---|---|---|
| total Lowry protein (mg) in S30 + PW/g fresh wt of cells | 64.6 ± 13.1 | 79.3 ± 23.8 | 80.8 ± 24.0 |
| nmol of GDP bound/mg of Lowry protein in the S30 + PW | 1.04 ± 0.40 | 1.66 ± 0.26 | 2.24 ± 0.19 |
| total nmol of EF-Tu present in S30 + PW/g fresh wt of cells | 67 | 132 | 181 |
| % of total extractable protein as EF-Tu in the S30 + PW | 4.3 | 6.9 | 9.3 |

*Isoelectric Focusing of Cell Lysates and Pure EF-Tu.* Two-dimensional isoelectric focusing–electrophoresis (Figure 1) clearly indicates only a single spot corresponding to EF-Tu, both in wild-type (MRE6000) and in the kirromycin-resistant (D2216) strain. Addition of pure crystalline, wild-type EF-Tu to the lysate sample (compare parts A and C of Figure 1) shows that this mutant spot is isoelectrically and electrophoretically indistingishable from wild-type EF-Tu. This is more clearly illustrated in the single-dimension isoelectric focusing patterns of the crystalline EF-Tu, purified from the mutant, parental, and wild-type strains (Figure 2). Each EF-Tu species shows only a single band, and the mixed sample gels show that the mutant EF-Tu $_{D2216}$ comigrates identically with wild-type and parental EF-Tu. These results show that kirromycin-resistant EF-Tu from strain D2216, like that from the other strains tested, was homogeneous as far as the sen-

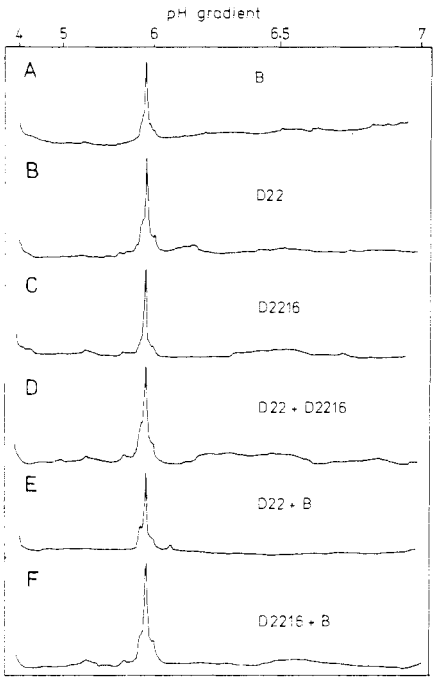


FIGURE 2: Scanning patterns of first-dimension isoelectric focusing gels of pure, crystalline EF-Tu prepared from various strains as indicated (see Materials and Methods). (A–C) Single samples; (D–F) mixed samples. Each gel contains the equivalent of 0.5 μg of EF-Tu from each strain.

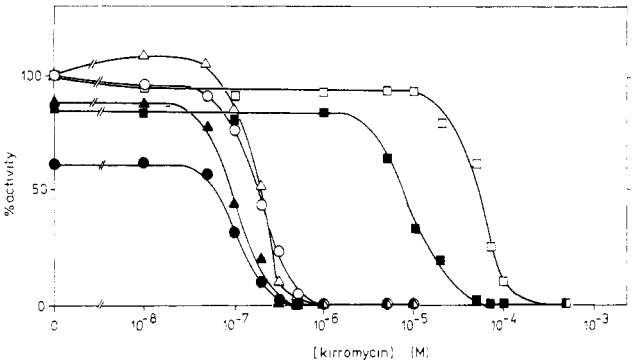


FIGURE 3: Effect of trypsinization on the ability of EF-Tu from various strains to support poly(U)-directed poly(phenylalanine) synthesis in the presence of increasing concentrations of kirromycin. Reaction mixtures contained in 75 μL 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 2.1 mM phosphoenolpyruvate, 0.01 μg of pyruvate kinase, 14 mM mercaptoethanol, 0.28 mM GTP, 0.29 mM ATP, 35 pmol of ribosomes, 100 pmol of [$^{14}$C]phenylalanine (sp act. 870 cpm/pmol), 0.4 μg of phenylalanyl-tRNA synthetase, 10 pmol of EF-Ts, 5 pmol of EF-G, 2 μg of long-chain poly(U), 72 μg of tRNA (Schwarz/Mann), kirromycin as indicated, 1.3% ethanol (carried over with the kirromycin), and between 12 and 17 pmol of EF-Tu purified from *E. coli* strains B (○, ●), D22 (△, ▲), and D2216 (□, ■), before (open symbols) or after (filled symbols) trypsinization for 30 min to 36K and 5K fragments as described under Materials and Methods. Incubation was for 10 min at 30 °C.

sitivity of the technique can distinguish.

*Mutant EF-Tu$_{D2216}$ in Binding Phe-tRNA$^{Phe}$ to the mRNA·Ribosome and in Poly(U)-Directed Poly(phenylalanine) Synthesis.* In both reactions, EF-Tu$_{D2216}$ was found not to differ in its effect significantly from the parental or wild-type elongation factor (experiments not shown).

*Effect of Trypsinization of Mutant and Wild-Type EF-Tu.* Trypsinization of EF-Tu was carried out under conditions which split the 43 225-dalton EF-Tu molecule into 36K and 5K fragments (Arai et al., 1980; Wittinghofer et al., 1980). The larger 36K fragment is further hydrolyzed into two fragments of about 19K and 17K only after a more prolonged trypsin treatment (Arai et al., 1980).

The mutation had no effect on the rate of digestion of EF-Tu·GDP. Addition of 50 μM kirromycin increased by 3–4-fold the rate of formation of the 36K fragment of wild-type EF-Tu$_B$·GDP, confirming other published results (Douglass & Blumenthal, 1979), but not that of mutant EF-Tu$_{D2216}$·GDP (not shown).

In has been shown (Arai et al., 1976; Jacobson & Rosenbusch, 1976, 1977) that the EF-Tu obtained by limited trypsin digestion conserves, in part, the ability to support several of the fundamental activities of the intact molecule. By use of carefully controlled conditions, all of the 43K native EF-Tu can be quantitatively converted into the 36K fragment. In poly(U)-directed poly(phenyalanine) synthesis (Figure 3), we found that after trypsinization 60–85% of the original activity was retained, in agreement with the observation of Jacobson & Rosenbusch (1977). In the presence of increasing concentrations of the antibiotic, there was a definite increase in sensitivity to kirromycin in all strains tested, by comparison with the native 43K EF-Tu. With EF-Tu from parental and wild-type strains, there is a 2-fold decrease in the concentration of kirromycin required to inhibit 50% of the activity. With mutant EF-Tu$_{D2216}$, the corresponding decrease is 7-fold.

*Endogenous GTPase Activity is Present in the Isolated Mutant EF-Tu$_{D2216}$.* In the course of purification and crystallization of the mutant EF-Tu$_{D2216}$, it was observed that in the absence of any effector the mutant elongation factor exhibited a higher background GTPase activity [~0.020 pmol/(min pmol of EF-Tu)] than occurs in our purified wild-type EF-Tu under comparable conditions [0.002 pmol/(min pmol EF-Tu)]. This was in spite of using two DEAE-Sephadex A-50 chromatography steps during the purification as recommended in Fischer et al. (1977). In wild-type strains, this chromatographic step eliminates several contaminating unspecific GTPases, one of which appears to elute on a shallow KCl gradient immediately before the EF-Tu peak (Fasano et al., 1978b). On repetition of this purification step on DEAE-Sephadex A-50 with the apparently electrophoretically pure crystalline EF-Tu$_{D2216}$, much of the GTPase activity could be separated from the EF-Tu peak (Figure 4). Nevertheless, a significant GTP hydrolysis was still associated with the mutant EF-Tu$_{D2216}$. This latter activity, unlike the original contaminant, could be strongly stimulated by the addition of ribosomes only, a phenomenon which is not detectable in wild-type EF-Tu. We also tested both the EF-Tu$_{D2216}$ and the preceding contaminant peak for the specificity of their GTPase activities by measuring their abilities to hydrolyze [$\gamma$-$^{32}$P]ATP. EF-Tu$_{D2216}$ showed no ATPase activity whatsoever, unlike the fractions of the preceding peak which could hydrolyze ATP at a rate ~30% that of their GTPase activity. Furthermore, the [$NH_4^+$] optimum for both the ATPase and GTPase activities of this preceding peak was <50 mM, in marked distinction from the endogenous GTPase activity of EF-Tu$_{D2216}$ where optimum was >200 mM. Subsequent experiments (see next section as well as the accompanying article) have confirmed that the persistant GTPase activity accompanying the EF-Tu$_{D2216}$ peak is endogenous to the mutant EF-Tu molecule and not a residual contamination.

*Influence of the Kirromycin-Resistant Mutation on Thermal Inactivation of EF-Tu.* Thermal inactivation profiles provide a useful tool to determine the extent of a conformational change in a protein induced by the addition of certain effectors. In Figure 5A,B are illustrated the thermal inactivation profiles for EF-Tu$_{D22}$·GDP and EF-Tu$_{D2216}$·GDP, incubated in the presence and absence of 50 μM kirromycin. As

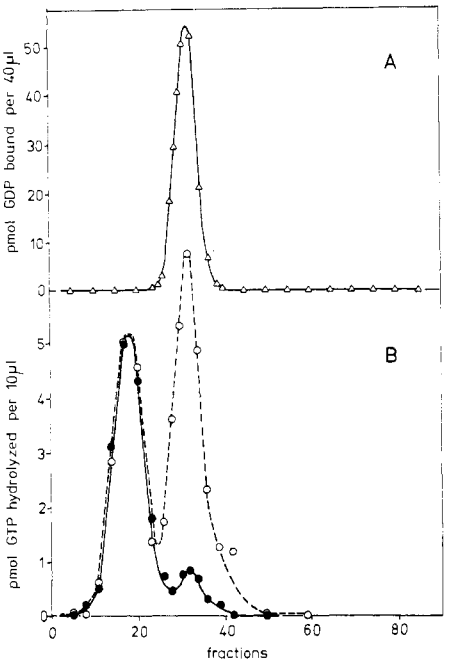


FIGURE 4: Purification of EF-$Tu_{D2216}$ by DEAE-Sephadex A-50 chromatography. (A) GDP-binding activity of eluate (▲); (B) GTPase activity of eluate in the presence (○) or absence (●) of ribosomes. A sample of ~5.5 mg of crystalline EF-$Tu_{D2216}$ was run on a 1 × 23 cm DEAE-Sephadex A-50 column, eluted with 2 × 250 mL of a linear 120–300 mM KCl gradient in a buffer also containing 60 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, and 20 µM GDP. Fractions of 4.5 mL were collected. GDP-binding activity was assayed by the addition of 60-µL aliquots of the fractions to a reaction mixture containing in 100 µL 50 mM imidazolium acetate, pH 7.5, 50 mM $NH_4Cl$, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 40 pmol of [$^3$H]GDP (sp act. 4300 cpm/pmol), followed by incubation for 30 min at 30 °C and filtration through nitrocellulose membrane filters as described under Materials and Methods. GTPase activity of the eluate was measured as described under Materials and Methods after addition of 10 µL of each fraction to an incubation mixture of a 75-µL final volume, which also contained 50 mM imidazolium acetate, pH 7.5, 60 mM $NH_4Cl$, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 160 pmol of [$^{32}$P]GTP (sp act. 4900 cpm/pmol) with (○) or without (●) 28 pmol of ribosomes. Incubation was for 20 min at 35 °C.

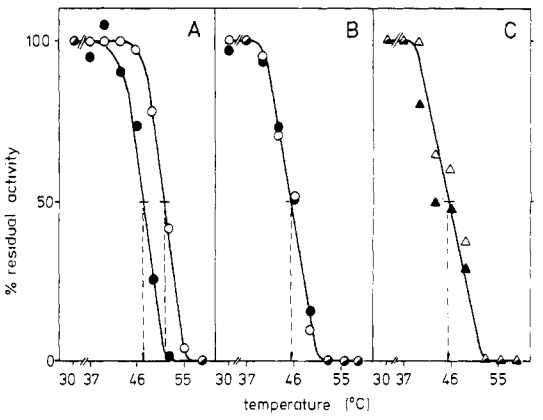


FIGURE 5: Thermal inactivation profiles for EF-Tu·GDP from *E. coli* strains D22 (A) and D2216 (B and C). The residual activity was measured by [$^3$H]GDP binding (A and B) or by the GTPase activity (C) in the presence (△) or absence (▲) of ribosomes only. Procedural details were as described under Materials and Methods. Thermal inactivation as measured by [$^3$H]GDP binding was carried out in the absence (●) or presence (○) of 50 µM kirromycin.

previously shown for wild-type EF-$Tu_B$·GDP (Chinali et al., 1977), addition of kirromycin considerably protects parental EF-$Tu_{D22}$·GDP from denaturation, increasing the $\theta_{1/2}$ from 47.2° to 51.4°. (The small differences in $\theta_{1/2}$ values between the present publication and that of Chinali et al. (1977) are due to the different incubation conditions used.) By contrast, there is no significant effect by the antibiotic on the $\phi_{1/2}$ for EF-$Tu_{D2216}$·GDP, which appears to be marginally lower (45.4°) than for parental EF-Tu·GDP under the same conditions.

Virtually identical thermal inactivation profiles are obtained for EF-$Tu_{D2216}$ when, instead of measuring its ability to bind [$^3$H]GDP, the residual activity is assayed by testing for the endogenous GTPase activity of mutant EF-$Tu_{D2216}$ in the absence of kirromycin (Figure 5C). Addition of ribosomes, which increases the activity of the system, does not change this picture. This result indicates that the endogenous GTPase activity, found uniquely in the mutant EF-$Tu_{D2216}$, is not due to a contaminating protein but to an activation of the normally latent catalytic center brought about by the mutation for kirromycin resistance.

## Discussion

Kirromycin blocks protein synthesis by inhibiting EF-Tu dissociation from the ribosome after enzymatic binding and the associated GTP hydrolysis (Wolf et al., 1977). It is for this reason that kirromycin sensitivity is phenotypically dominant over kirromycin resistance (Fischer et al., 1977). EF-Tu being in large excess over the number of ribosomes in vivo (Kjeldgaard & Gausing, 1974; Furano, 1975), it requires only a small proportion of the total EF-Tu present to be kirromycin sensitive for all the ribosomes to be blocked (Fischer et al., 1977). This would be particularly true with polysomes, where only one kirromycin-sensitive EF-Tu could block the activity of several ribosomes. On the basis of such an argument, it must be assumed that for the mutant, kirromycin-resistant phenotype to be expressed either one of the two *tuf* genes in *E. coli* or its product is inactive in protein synthesis, or that both genes are similarly mutated.

The preliminary studies of Fischer et al. (1977), in fact, indicated that kirromycin resistance could be transferred from the mutant strain D2216 to the parental strain D22 via the *tufB* gene. This is in contrast to the kirromycin-resistant mutants of the group of Bosch (Van der Klundert et al., 1978) where it is *tufA* that confers resistance to the antibiotic. Further genetic studies will have to be completed before we can reach a final conclusion as to the genetic basis of kirromycin resistance in strain D2216. The lower in vivo quantity of EF-Tu in the mutant strain is not necessarily related to the activity of only one of the *tuf* genes, but it can rather be a consequence of the slower growth rate [see Pedersen et al. (1978)]. In this regard, we have determined that in a recombinant strain of *E. coli* containing at least six cloned *tufB* genes [Bernardi & Bernardi (1979) and personal communication], the in vivo quantity of EF-Tu was not greater than in normal wild-type strains and corresponded closely with the growth rate of this modified strain (R. Ivell, unpublished observations). Thus, whether the in vivo quantity of EF-Tu is the result or a cause of these slower growth rates remains an open question.

The isolectric focusing study indicates that the kirromycin-resistant EF-Tu present in strain D2216 is indistinguishable from wild-type EF-Tu and is homogeneous. In other mutant strains, such as HAK 88 (Pedersen et al., 1976a; Lemaux & Miller, 1978) or the kirromycin-resistant *Bacillus subtilis* (Smith & Paress, 1978), the mutant EF-Tu exhibits an altered net charge, manifested as a shift in the isoelectric focusing band by comparison with wild-type, parental EF-Tu. The group of Bosch (L. Bosch, personal communication) has also found two isoelectrically distinct EF-Tu species in a kirro-

mycin-resistant mutant of *E. coli*, where *tufB* gives rise to an inactive product. In the light of these results, the finding that EF-$Tu_{D2216}$ is isoelectrically homogeneous strongly suggests that only one mutated *tuf* gene product is present and that the mutation, while affecting the kirromycin resistance of the *EF*-Tu, does not alter its total net charge. If a second *tuf* gene product is present it must be degraded into smaller fragments, which cannot be identified in the gel system used. Direct support for the homogeneity of the mutant EF-$Tu_{D2216}$ is also given by the fingerprints of total tryptic digests of parental and mutant EF-Tu (Fischer et al., 1977): only one spot in the EF-$Tu_{D2216}$ digest is shifted in position by comparison with the parental digest, and no shadow of the original parental spot was detectable. Such a change indicates a homogeneous protein differing only at a single site from the parental elongation factor.

In poly(U)-directed poly(phenylalanine) synthesis, as well as in enzymatic binding of Phe-$tRNA^{Phe}$ to ribosomes, the isolectrically pure, crystalline EF-$Tu_{D2216}$ appears to function like wild-type or parental EF-Tu (EF-$Tu_{D22}$ seems identical with wild-type EF-Tu by a variety of biochemical criteria). Nor does the mutation appear to influence the conformation of EF-Tu·GDP accessible to partial trypsin hydrolysis, a technique which has been recently shown to be extremely sensitive in distinguishing the different conformations of EF-Tu induced by guanine nucleotides or by kirromycin (Douglass & Blumenthal, 1979). The altered affinity for kirromycin of the mutant EF-$Tu_{D2216}$ is expressed by the inability of kirromycin to increase the rate of trypsinization, in contrast to the effect of the antibiotic on wild-type EF-Tu, a result paralleled in the thermal inactivation profiles. Limited trypsinization does not appear to influence greatly the principal functions of the wild-type elongation factor, up to 85% of the original activity being retained in poly(phenylalanine) synthesis (this article; Jacobson & Rosenbusch, 1977).

One property that does appear to be influenced by trypsinization is the interaction between EF-Tu and the antibiotic, the trypsinized molecule having a higher sensitivity to the inhibiting effects of kirromycin in polypeptide synthesis. This is most marked in the mutant EF-$Tu_{D2216}$, where there is a 7-fold increase in sensitivity.

A very important finding of this study is that the mutant EF-$Tu_{D2216}$ exibits a GTPase activity in the absence of any other effector. The stimulation by ribosomes, its specificity for GTP, the thermal denaturation profiles, and, most important, the characterization of this activity reported in the following paper exclude the possibility that it can be attributable to a contaminating protein. Therefore our study represents an additional demonstration that EF-Tu is a GTPase. Via a mutation its activity can now be revealed without the aid of any exogenous agent.

## Acknowledgments

We are particularly indebted to Dr. G. Sander for critically reading the manuscript. Dr. E. Fischer (Institut für Biologie II, Lehrbereich Mikrobiologie, Universität Tübingen, Federal Republic of Germany) carried out some preliminary experiments concerning the trypsinization of EF-Tu.

## References

Arai, K., Nakamura, S., Arai, T., Kawakita, M., & Kaziro, Y. (1976) *J. Biochem. (Tokyo) 79*, 69–83.

Arai, K., Clark, B. F. C., Duffy, L., Jones, M. D., Kaziro, Y., Laursen, R. A., L'Italien, J., Miller, D. L., Nagarkatti, S., Nakamura, S., Nielsen, K. M., Petersen, T. E., Takahashi, K., & Wade, M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1326–1330.

Bernardi, A., & Bernardi, F. (1979) *Eur. J. Biochem. 95*, 391–398.

Chinali, G., & Parmeggiani, A. (1973) *Eur. J. Biochem. 32*, 463–472.

Chinali, G., Wolf, H., & Parmeggiani, A. (1977) *Eur. J. Biochem. 75*, 55–65.

Douglass, J., & Blumenthal, T. (1979) *J. Biol. Chem. 254*, 5383–5387.

Fasano, O., Bruns, W., Crechet, J.-B., Sander, G., & Parmeggiani, A. (1978a) *Eur. J. Biochem. 89*, 557–565.

Fasano, O., Parlato, G., Cocchiararo, M., & Bocchini, V. (1978b) *Bull. Mol. Biol. Med. 3*, 54–68.

Fischer, E., Wolf, H., Hantke, K., & Parmeggiani, A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4341–4345.

Furano, A. V. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4780–4784.

Furano, A. V. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3104–3108.

Herendeen, S. L., VanBogelen, R. A., & Neidhardt, F. C. (1979) *J. Bacteriol. 139*, 185–194.

Jacobson, G. R., & Rosenbusch, J. P. (1976) *Biochemistry 15*, 5105–5110.

Jacobson, G. R., & Rosenbusch, J. P. (1977) *Eur. J. Biochem. 77*, 409–417.

Jaskunas, S. R., Lindahl, L., Nomura, M., & Burgess, R. R. (1975) *Nature (London) 257*, 458–462.

Kjeldgaard, N. D., & Gausing, K. (1974) in *Ribosomes* (Nomura, M., Tissières, A., & Lengyel, P., Eds.) pp 369–392, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Lemaux, P. G., & Miller, D. L. (1978) *Mol. Gen. Genet. 159*, 47–55.

Lowry, O. G., Rosebrough, N. J., Farr, A. L., & Randall, R. (1951) *J. Biol. Chem. 193*, 265–275.

Miller, D. L., & Weissbach, H. (1977) in *Molecular Mechanisms of Protein Biosynthesis* (Weissbach, H., & Pestka, S., Eds.) pp 332–373, Academic Press, New York.

Monner, D. A., Jonsson, S., & Boman, H. G. (1971) *J. Bacteriol. 107*, 420–432.

Noll, M., & Noll, H. (1976) *J. Mol. Biol. 105*, 111–130.

Normark, S., Boman, H. G., & Matsson, E. (1969) *J. Bacteriol. 97*, 1334–1342.

O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007–4021.

Parmeggiani, A., & Sander, G. (1980) in *Topics in Antibiotic Chemistry* (Sammes, P. G., Ed.) pp 159–221, Wiley, Baffins Lane, England.

Parmeggiani, A., Wolf, M., & Chinali, G. (1976) in *Ribosomes and RNA Metabolism* (Zelinka, J., & Balan, J., Eds.) Vol. 2, pp 283–290, Publishing House of the Slovak Academy of Sciences, Bratislava.

Pedersen, S., Blumenthal, R. M., Reeh, S., Parker, J., Lemaux, P., Laursen, R. A., Nagarkatti, S., & Friesen, J. D. (1976a) *Proc. Natl. Acad. Sci. U.SA. 73*, 1698–1701.

Pedersen, S., Reeh, S., Parker, J., Watson, R. J., Friesen, J. D., & Fiil, N. P. (1976b) *Mol. Gen. Genet. 144*, 339–343.

Pedersen, S., Bloch, P. L., Reeh, S. V., & Neidhart, F. C. (1978) *Cell (Cambridge, Mass.) 14*, 179–190.

Sander, G., Marsh, R. C., Voigt, J., & Parmeggiani, A. (1975) *Biochemistry 14*, 1805–1814.

Sander, G., Okonek, M., Crechet, J. B., Ivell, R., Bocchini, V., & Parmeggiani, A. (1979) *FEBS Lett. 98*, 111–114.

Smith, I., & Paress, P. (1978) *J. Bacteriol. 135*, 1107–1117.

Van de Klundert, J. A. M., Den Turk, E., Borman, A. H., Van der Meide, P. H., & Bosch, L. (1977) *FEBS Lett. 81*, 303–307.

Van de Klundert, J. A. M., Van der Meide, P. H., Van de Putte, P., & Bosch, L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4470–4473.

Wittinghofer, A., Frank, R., & Leberman, R. (1980) *Eur. J. Biochem. 108*, 423–431.

Wolf, H., Chinali, G., & Parmeggiani, A. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4910–4914.

Wolf, H., Chinali, G., & Parmeggiani, A. (1977) *Eur. J. Biochem. 75*, 67–75

# 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 [$\gamma$-$^{32}$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).

† From the Laboratoire de Biochimie (Laboratoire Associé No. 240 du Centre National de la Recherche Scientifique), Ecole Polytechnique, 91128 Palaiseau Cedex, France (O.F. and A.P.), and Cattedra di Chimica, II[a] Facoltà di Medicina e Chirurgia, Università di Napoli, 80131 Napoli, Italy (O.F.). *Received November 26, 1979; revised manuscript received August 13, 1980.* This work was supported by grants from the Délégation Générale à la Recherche Scientifique et Technique (No. 76.1.1186 and 78.7.1097) and NATO (No. 1706) and by the Commissariat à l'Energie Atomique.

[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.

0006-2960/81/0420-1361$01.25/0 