Elongation Factor Tu D138N, a Mutant with Modified Substrate Specificity, as a Tool To Study Energy Consumption in Protein Biosynthesis†

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ABSTRACT: Substitution Asp138→Asn changes the substrate specificity of elongation factor (EF) Tu from GTP to XTP [Hwang & Miller (1987) *J. Biol. Chem. 262*, 13081–13085]. This mutated EF-Tu (EF-Tu D138N) was used to show that 2 XTP molecules are hydrolyzed for each elongation cycle [Weijland & Parmeggiani (1993) *Science 259*, 1311–1313]. Here we extend the study of the properties of this EF-Tu mutant and its function in the elongation process. In poly(U)-directed poly(phenylalanine) synthesis, the number of peptide chains synthesized using EF-Tu D138N·XTP was 30% higher than with EF-Tu wild type (wt)·GTP. However, since in the former case the average peptide chain length was correspondingly reduced, the number of the residues incorporated turned out to be nearly the same in both systems. The K'_d values of the XTP and XDP complexes of EF-Tu D138N were similar to those of the GTP and GDP complexes of EF-Tu wt. The extent of leucine misincorporation and the kirromycin effect were also comparable to those in the EF-Tu wt/GTP system. The hydrolysis of two XTP molecules, very likely as part of two EF-Tu D138N·XTP complexes, for each elongation cycle was found to be independent of (i) $MgCl_2$ concentration, (ii) ribosome concentration, and (iii) temperature (5–40 °C). With rate-limiting amounts of XTP the K'_m of its XTPase activity corresponded to the K'_m for XTP of poly(phenylalanine) synthesis (0.3–0.6 μM). This correlation strongly suggests that both XTP molecules are involved in the basic mechanism of the EF-Tu-mediated binding of aminoacyl-tRNA to the ribosome and do not participate in idling activities. With concentrations of $MgCl_2$ higher than 9 mM, the EF-G-dependent GTPase became strongly uncoupled from poly(phenylalanine) synthesis, whereas the XTPase activity of EF-Tu started to be uncoupled at $MgCl_2$ concentrations higher than 12 mM. The results of this work prove that the EF-Tu D138N/XTP system is a powerful tool for analyzing bioenergetic aspects of protein biosynthesis.

The energy consumption in the elongation cycle of protein biosynthesis is associated with the function of the elongation factors Tu (EF-Tu)[1] and G (EF-G) and plays a crucial role for the rate of amino acid incorporation and its accuracy (Dix *et al.*, 1990; Kurland *et al.*, 1990). Recently, EF-Tu D138N, a mutant with a modified substrate specificity using XTP instead of GTP (Hwang & Miller, 1987; Weijland & Parmeggiani, 1993), was reported to allow the direct measurement of the energetics dependent on EF-Tu during the elongation cycle. The fact that both EF-Tu and EF-G are GTPases, whose activities can also be triggered by the ribosome independently of elongation (uncoupled GTPase activity), created in the past considerable difficulties for the estimation of their specific activities (Ehrenberg *et al.*, 1990; Thompson & Karim, 1982; Bensch *et al.*, 1991; Chinali & Parmeggiani, 1980). The possibility to have a system that allows the separate evaluation of the hydrolytic activity dependent on EF-Tu and EF-G was therefore of fundamental importance. In order to resolve this problem, the use of EF-Tu D138N/XTP was supported by the consideration that according to the structure of XTP and GTP this system should behave bioenergetically as the EF-Tu wt/GTP system. Indeed, EF-Tu D138N/XTP made it possible to show that two XTP molecules are hydrolyzed for each elongation cycle, as was earlier proposed by Ehrenberg *et al.* (1990) for the EF-Tu wt/GTP system on the basis of kinetic data.

Considering the importance of this finding for the basic mechanism of elongation, the need for a detailed characterization of EF-Tu D138N for assessing unequivocally the ability of this mutant to substitute for EF-Tu wt was evident. Therefore, the partial characterization of EF-Tu D138N, reported previously (Weijland & Parmeggiani, 1993), has now been extended to a larger spectrum of experimental conditions. The results obtained confirm that this EF-Tu mutant is a valid substitute of EF-Tu wt for studying the energetics of elongation. Its use has allowed the analysis of additional aspects of this process that were either obscure or incompletely understood.

MATERIALS AND METHODS

EF-Tu D138N was constructed by site-directed mutagenesis of the encoding *tufA* gene cloned in a pEMBL9+, using the

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[1] Abbreviations: EF-Tu, elongation factor Tu; EF-TuAr, the product of the *tufA* gene encoding a kirromycin-resistant EF-Tu mutant carrying substitution A375T; EF-Ts, elongation factor Ts; EF-G, elongation factor G; A-site, the ribosomal site accepting aminoacyl-tRNA from the ternary complex and binding puromycin-unreactive peptidyl-tRNA; P-site, the ribosomal site binding puromycin-reactive peptidyl-tRNA; E-site, the ribosomal site to which discharged tRNA is shifted during the translocation of peptidyl-tRNA from the A- to the P-site; PMSF, phenylmethanesulfonyl fluoride; ME, 2-mercaptoethanol; TCA, trichloroacetic acid; wt, wild type.

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gapped duplex method (Parmeggiani *et al.*, 1987) and the oligodeoxynucleotide CAAATGCAACATGGTT (Parlato & Parmeggiani, 1988). The mutated *tufA* gene was transferred in the *tac* vector pTTQ18 and expressed in the *recA*$^-$ *Escherichia coli* PM1455 strain containing only one active *tuf* gene (*tufAr*), whose product is resistant to kirromycin (Van der Meide *et al.*, 1983). After induction with IPTG, the cells were collected, washed, and stored at −20 °C.

Isolation and Purification of EF-Tu D138N. The procedure for the isolation and purification of this EF-Tu mutant made use of the properties of kirromycin to increase the negative net charge of ET-Tu and compete with elongation factor Ts for binding to EF-Tu, as already reported (Swart *et al.*, 1987; Jacquet & Parmeggiani, 1989; Anborgh *et al.*, 1991). The *recA*$^-$ *E. coli* PM1455 cells containing only one active *tuf* gene, *tufAr*, encoding the kirromycin-resistant EF-TuAr were transformed with pTTQ18-*tuf*AD138N and induced with IPTG. These cells (20 g) were sonicated in buffer A [50 mM Tris-HCl, pH 7.6, 7 mM $MgCl_2$, 50 mM KCl, and 7 mM 2-mercaptoethanol (ME)] containing 1 mM PMSF and 15% glycerol. After dialysis against buffer A, the supernatant obtained after centrifugation at 30000*g* was used for a first chromatographic step on DEAE-Sepharose Fast Flow (Pharmacia), carried out with a linear 50–250 mM KCl gradient in buffer A to isolate from the other proteins the fraction containing both the EF-Tu D138N and EF-TuAr complexes with EF-Ts. The concentrated EF-Tu·EF-Ts pool was incubated with 50 μM kirromycin in buffer A and then applied to a second DEAE-Sepharose Fast Flow column. In a linear gradient of 50–250 mM KCl in buffer A containing 10 μM kirromycin, EF-TuAr·EF-Ts and EF-Tu D138N·kirromycin emerge as two completely separated peaks. After concentration of the most active fractions, EF-Tu D138N·kirromycin was passed through a long (200 × 1 cm) AcA54 column. The active peak containing pure EF-Tu D138N·kirromycin was concentrated and dialyzed for 60 h against a 10-fold excess of EF-Ts in order to remove kirromycin. A third DEAE-Sepharose Fast Flow chromatographic step was performed to separate EF-Tu D138N·EF-Ts from EF-Tu D138N·kirromycin. The resulting pure EF-Tu D138N·EF-Ts was stored at −20 °C in buffer A containing 50% glycerol. EF-Tu D138N free from EF-Ts was obtained *via* a successive DEAE-Sepharose Fast Flow chromatographic step using a 0–250 mM KCl gradient in standard buffer A containing 0.5 μM XDP. Prior to loading, the EF-Tu·EF-Ts solution had been preincubated with 10 μM XDP in buffer A.

Poly(U)-Directed Poly(phenylalanine) Synthesis. This reaction was started by adding to mix I an equal volume of mix II. Mix I contained, in buffer B (same as standard buffer A except for 70 mM NH_4Cl replacing KCl), ribosomes, poly(U), and *N*-acetyl-Phe-tRNA^{Phe} or tRNA^{Phe} and was kept for 40 min at 0 °C prior to the use. Mix II contained in buffer B the elongation factors, the diverse nucleotides, and the tRNA charging system and was preincubated for 10 min at 37 °C. The average-chain length was calculated from the [^{3}H]/[^{14}C] ratio of the [^{3}H]phenylalanine and *N*-acetyl-[^{14}C]phenylalanine incorporated, while the number of chains was calculated from the ratio between the picomoles of *N*-acetyl-[^{14}C]-phenylalanine incorporated and the picomoles of ribosomes. The phenylalanine incorporated into the polypeptide chain was measured by using the hot trichloroacetic acid (TCA) precipitation method (Weijland & Parmeggiani, 1993). For other technical details, see the legends to the figures.

GTPase Activity and Other Methods. The GTP or XTP hydrolysis was determined by measuring the $^{32}P_i$ liberated from [γ-^{32}P]GTP or [γ-^{32}P]XTP via the charcoal method (Weijland & Parmeggiani, 1993). [γ-^{32}P]GTP was obtained from Du Pont/NEN, and [γ-^{32}P]XTP was synthesized by the method of Glynn and Chappel (1964). The specific activity of the [γ-^{32}P]XTP synthesized was checked by carrying out independent dilution experiments with cold XTP. The K_d was determined as reported (Fasano *et al.*, 1982). Protein concentration was measured with the Bradford method (Bradford, 1976), using bovine serum albumin as standard.

RESULTS

Purification and Properties of EF-Tu D138N. We would like to emphasize a few aspects of the isolation and purification of EF-Tu D138N. This mutated EF-Tu could be extracted in a soluble form only in complex with EF-Ts. In the cell and during cell extraction EF-Ts appeared to protect EF-Tu D138N as a chaperon-like molecule (Fasano & Parmeggiani, 1976; Martin *et al.*, 1992; Rothman *et al.*, 1989). The fraction of EF-Tu D138N (>95%) nonassociated with EF-Ts formed insoluble inactive precipitates, very likely as a consequence of an incomplete folding caused by (i) the low concentration of XTP (XDP), if any, in the cell and (ii) the inability of EF-Tu D138N to interact with GTP or GDP. Nucleotide-free EF-Tu is known to be very unstable and can be stabilized by EF-Ts or kirromycin (Fasano & Parmeggiani, 1976). For this reason, we carried out the separation of EF-Tu D138N from EF-TuAr, both in complex with EF-Ts, in the presence of kirromycin (Swart *et al.*, 1987; Anborgh *et al.*, 1991), in order to have the constant presence of a stabilizer of the nucleotide-free EF-Tu. The yield of EF-Tu D138N bound to EF-Ts and EF-Tu D138N bound to kirromycin was low, 0.1–0.2 mg and about 0.5 mg/20 g of cells, respectively, but the results were reproducible, as tested in 10 different preparations. The reasons for the low recovery of the EF-Tu D138N·EF-Ts complex extracted from the cell were very likely (i) the low amount of EF-Ts in the cell, corresponding to a few percent the amount of EF-Tu, and (ii) the competition between EF-Tu D138N and the chromosomic wild-type EF-Tu for binding to EF-Ts. We have tried to use pGEX vectors producing EF-Tu D138N fused with glutathione *S*-transferase, but no improvement could be obtained, the largest part of the resulting product being insoluble (A Weijland and P. H. Anborgh, unpublished results). EF-Tu D138N in complex with EF-Ts or kirromycin was stable for at least 1 year if kept at −20 °C in buffer A containing 50% glycerol.

Comparison between the Activities of EF-Tu D138N and EF-Tu. As reported earlier (Weijland & Parmeggiani, 1993), EF-Tu D138N requires the specific presence of XTP for translation *in vitro*. No other purine nucleotide was able to substitute for XTP (data not shown). The dissociation constants (K'_d) of its complexes with XTP (750 nM) and XDP (0.9 nM) were similar to those of EF-Tu wt for GTP (530 nM) and GDP (0.8 nM). The same holds true for the dissociation rates of XTP. $t_{50\%}$ values of 80 and 70 min were determined for EF-Tu wt·kirromycin and EF-Tu D138N·kirromycin, respectively. Previously, we had shown that the rates of poly(U)-directed poly(phenylalanine) synthesis with EF-Tu D138N·XTP and EF-Tu wt·GTP are remarkably similar (Weijland & Parmeggiani, 1993). As a further characterization of this activity, we determined the average chain length and the number of chains formed catalyzed by EF-Tu D138N and EF-Tu wt in poly(phenylalanine) synthesis (Figure 1). Interestingly, the chains were found to be about a third longer in the case of EF-Tu wt. However, in the EF-Tu D138N/XTP system the decrease in the chain length was

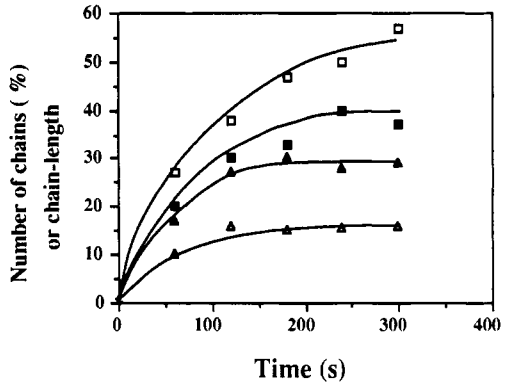

FIGURE 1: Average length (squares) and number (triangles) of the poly(phenylalanine) chains in the EF-Tu D138N-XTP system (closed symbols) and the EF-Tu wt-GTP system (open symbols). In mix I, 70S ribosomes (1 μM) were incubated in 250 μL of buffer B with 150 μg of poly(U) and 1.2 μM [^{14}C]-*N*-acetyl-Phe-tRNA^{Phe} (0.1 Ci/mmol). Mix II contained, in 250 μL of buffer B, 1 mM ATP, 100 μM GTP, 0.05 μM EF-Tu wt or D138N, 10 μM XTP (only for EF-Tu D138N), 0.2 μM EF-Ts, 8 mM [^{3}H]phenylalanine (0.1 Ci/mmol), 5 μM tRNA^{Phe}, 0.015 μM purified phenylalanyl-tRNA synthetase, and 0.1 μM EF-G. At the given times 30-μL samples were taken from the reaction mixture and incubated at 30 °C, and the ^{3}H and ^{14}C counts incorporated in the poly(phenylalanine) chain were measured (see Materials and Methods). The standard error for the single experimental values, as calculated in independent experiments, lies within 6%. This variance is also valid for the experiments reported in Figures 2–6 and 8.

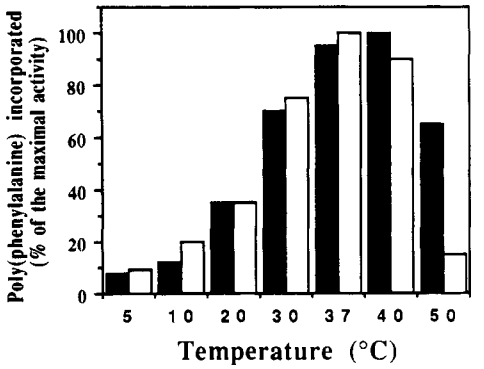

FIGURE 2: Rate of poly(phenylalanine) synthesis as a function of the temperature. The 50-μL reaction mixtures (25 μL of mix I + 25 μL of mix II) were incubated at the indicated temperatures. In mix I, 1 μM 70S ribosomes were incubated with 15 μg of poly(U) and 3 μM tRNA^{Phe} in buffer B. Mix II contained, in standard buffer B, 1 mM ATP, 100 μM GTP, 0.05 μM EF-Tu wt or D138N, 10 μM XTP (only for EF-Tu D138N), 0.2 μM EF-Ts, 8 mM [^{3}H]-phenylalanine (0.1 Ci/mmol), 5 μM tRNA^{Phe}, 0.015 μM purified phenylalanyl-tRNA synthetase, and 0.1 μM EF-G. After a 20-min incubation, 30-μL samples were taken to measure the amount of phenylalanine incorporated into the peptide chain. The EF-Tu wt-GTP system (solid bars) and the EF-Tu D138N-XTP system (open bars) are shown. The background values in a system omitting EF-Tu subtracted for each point corresponded to 2, 4, 4, 3, 4, and 8 pmol of phenylalanine incorporated/pmol of EF-Tu, respectively.

compensated by a similar increase in the number of polypeptide chains, explaining the comparable rate of phenylalanine incorporation.

The temperature dependence of both systems was similar up to 37–40 °C (Figure 2). At higher temperatures, the velocity of the poly(phenylalanine) synthesis obtained with EF-Tu D138N/XTP decreased more rapidly than that with EF-Tu wt. At 50 °C, EF-Tu D138N showed only 10–15% of the maximal activity, whereas EF-Tu wt was still 60–65% as active.

The accuracy of polypeptide synthesis observed with EF-Tu D138N, as assessed from the misincorporation of leucine

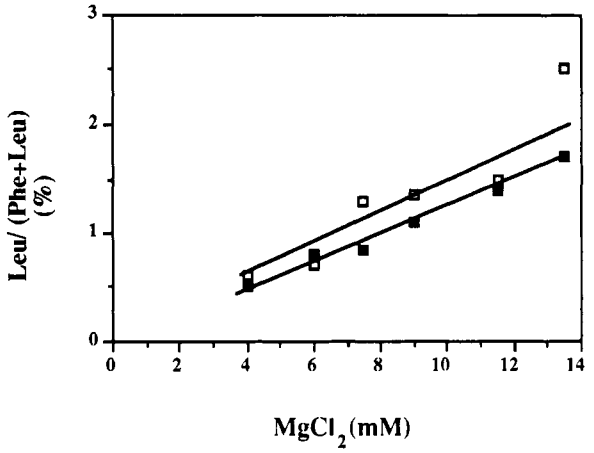

FIGURE 3: Accuracy of protein biosynthesis with EF-Tu wt and D138N as a function of different $MgCl_2$ concentrations. The reactions mixtures (50 μL; 25 μL of mix I + 25 μL of mix II) were incubated in buffer B with $MgCl_2$ concentrations as indicated. Mix I contained 1 μM 70S ribosomes, 15 μg of poly(U), and 3 μM tRNA^{Phe}. Mix II contained 1 mM ATP, 100 μM GTP, 0.05 μM EF-Tu wt or D138N, 10 μM XTP (only for EF-Tu D138N), 0.2 μM EF-Ts, 5 mg/mL crude tRNA, 0.015 μM leucyl-tRNA synthetase, 8 mM [^{3}H]leucine (0.5 Ci/mmol), 8 mM [^{14}C]phenylalanine (0.1 Ci/mmol), 0.015 μM purified phenylalanyl-tRNA synthetase, and 0.1 μM EF-G. After a 20-min incubation at 30 °C, 30-μL samples were taken to measure the leucine misincorporation in the poly(phenylalanine) peptide chain. EF-Tu wt (□) and EF-Tu D138N (■) experiments are shown. The background values subtracted were comparable to those of Figure 2.

in the poly(U)-dependent poly(phenylalanine) synthesis, was comparable to that of the EF-Tu wt, up to 14 mM $MgCl_2$ concentration (Figure 3). Also, the sensitivity of the EF-Tu D138N-dependent poly(phenylalanine) synthesis toward inhibition by kirromycin was the same as that of EF-Tu wt (not shown).

EF-Tu D138N XTPase Activity: Hydrolysis of Two XTP's during the Elongation Cycle Is Independent of Ribosome Concentration and Temperature. The stimulation by kirromycin of the XTPase of EF-Tu D138N *vs* that of the GTPase of EF-Tu wt was only half as high (Figure 4). However, the enhancing effects of kirromycin on the activity of the two EF-Tu species in the presence of nonprogrammed ribosomes were comparable.

On the basis of kinetic studies, a pentameric complex between one aminoacyl-tRNA molecule and two EF-Tu·GTP complexes was proposed to be the carrier of aminoacyl-tRNA in elongation (Ehrenberg *et al.*, 1990). However, Bensch *et al.* (1991) and Abrahams *et al.* (1991), using various biochemical and physical methods, confirmed the existence of a ternary complex under a broad spectrum of conditions. Recently, Bilgin and Ehrenberg (1993) have reported that this pentameric complex is stable at 37 °C but not at 20 °C. We therefore tested whether the 2:1 stoichiometry between the EF-Tu-mediated XTP hydrolysis and the poly(phenylalanine) synthesized was dependent on the temperature. As shown in Figure 5, no deviation was found for temperatures between 5 and 40 °C. Thus, two XTP molecules are consumed during the EF-Tu·ribosome interaction, even under conditions in which the elongation rate is strongly decreased by low temperatures. An additional problem is represented by the fact that only some of the ribosomes are active in translation. The ribosomes with an active peptidyltransferase center form at most 50–60% of the total population (Chinali & Parmeggiani, 1980). Wagner *et al.* (1992) reported that the fraction of ribosomes with homogeneous activity in poly(phenylalanine) synthesis is even lower, probably only 10–15%, as was estimated

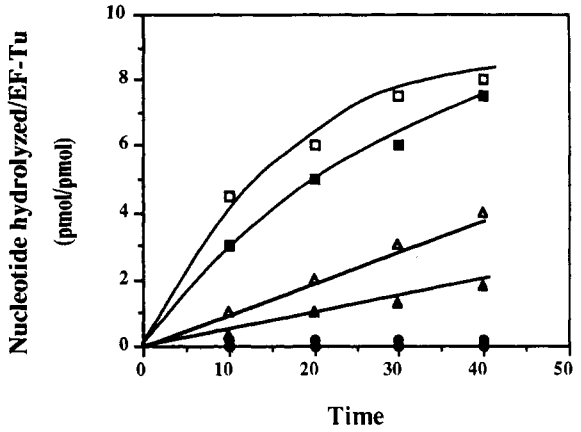

FIGURE 4: Stimulation of the XTPase of EF-Tu D138N (closed symbols) and the GTPase of EF-Tu wt (open symbols) by kirromycin and ribosomes. The intrinsic activities are indicated by circles, the stimulation by kirromycin by triangles, and stimulation by kirromycin plus ribosomes by squares. The reactions were carried out at 30 °C in buffer B (100 μL) with 1 μM kirromycin, 1 μM ribosomes, 0.05 μM EF-Tu wt or D138N, and 10 μM [γ-^{32}P]GTP (0.1 Ci/mol, EF-Tu wt, open symbols) or [γ-^{32}P]XTP (0.1 Ci/mol, EF-Tu D138N, closed symbols). The reactions were started by the addition of EF-Tu. At the times indicated, 20-μL samples were taken from the reaction mixture and the $^{32}P_i$ liberated was measured by the charcoal method (Weijland & Parmeggiani, 1993). The average background values subtracted in a system omitting EF-Tu corresponded to about 0.05 pmol of nucleotide hydrolyzed/pmol of EF-Tu.

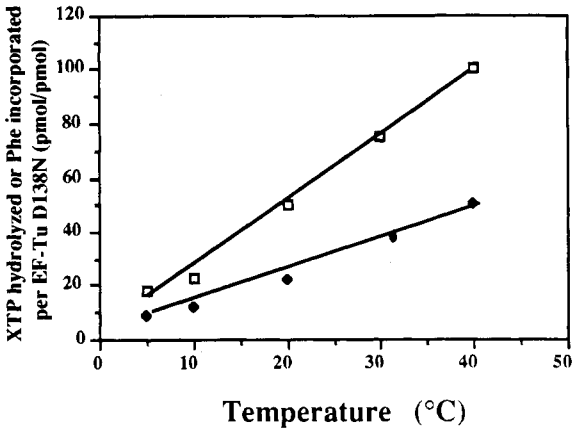

FIGURE 5: Temperature does not influence the stoichiometric ratio between XTP hydrolyzed and phenylalanine incorporated. The 50-μL reaction mixtures (25 μL of mix I + 25 μL of mix II) were incubated in buffer B at the indicated temperatures. Mix I contained 1 μM 70S ribosomes, 15 μg of poly(U), and 3 μM tRNA^{Phe}. Mix II contained 1 mM ATP, 100 μM GTP, 0.05 μM EF-Tu, D138N, 20 μM [γ-^{32}P]XTP (0.1 Ci/mmol), 0.2 μM EF-Ts, 8 mM [^{3}H]-phenylalanine (0.1 Ci/mmol), 5 μM tRNA^{Phe}, 0.015 μM purified phenylalanyl-tRNA synthetase, and 0.1 μM EF-G. After a 20-min incubation, 20-μL samples were withdrawn to measure the XTP hydrolysis (□) and phenylalanine incorporation (◆).

from the initial burst phase of the phenylalanine incorporation. Such heterogeneity could favor side reactions, whose idle GTPases cannot be corrected by specific blanks. We therefore examined whether the 2:1 stoichiometry between XTP hydrolysis and phenylalanine incorporated may have varied as a function of ribosome concentration under conditions of constant rate-limiting amounts of EF-Tu D138N. As shown in Figure 6, also in this case we were unable to find any variation of the 2:1 stoichiometric ratio.

Coupling of the EF-Tu XTPase and EF-G GTPase Activities with Poly(phenylalanine) Synthesis. Chinali and Parmeggiani (1980) concluded that the apparent K'_m for GTP of steady-state poly(phenylalanine) synthesis at low magnesium concentration and rate-limiting amounts of EF-G

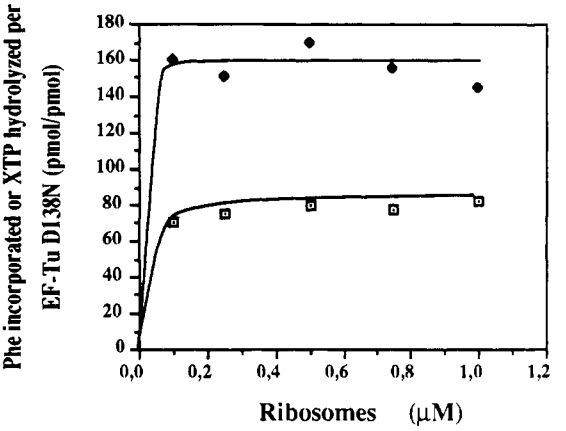

FIGURE 6: Increasing the concentration of ribosomes does not influence the final stoichiometry between XTP hydrolysis and phenylalanine incorporation. The 50-μL reaction mixtures (25 μL of mix I + 25 μL of mix II) were incubated with the ribosome concentrations as indicated. Mix I contained, in buffer B, 70S ribosomes (micromolar), tRNA^{Phe} (micromolar), and poly(U) (micrograms) (μg) in a 1:3:15 ratio as indicated. Mix II contained, in buffer B, 1 mM ATP, 100 μM GTP, 20 μM [γ-^{32}P]XTP (0.1 Ci/mmol), 0.05 μM EF-Tu D138N, 0.2 μM EF-Ts, 8 mM [^{3}H]-phenylalanine (0.1 Ci/mmol), 5 μM tRNA^{Phe}, 0.015 μM purified phenylalanyl-tRNA synthetase, and 0.1 μM EF-G. The reactions were carried out at 30 °C. After a 20-min incubation, 20-μL samples were withdrawn to measure the XTP hydrolysis (◆) and the phenylalanine incorporation (⊡). The average background values subtracted corresponded to 15 pmol of XTP hydrolyzed/pmol of EF-Tu D138N and 1 pmol of phenylalanine incorporated/pmol of EF-Tu D138N.

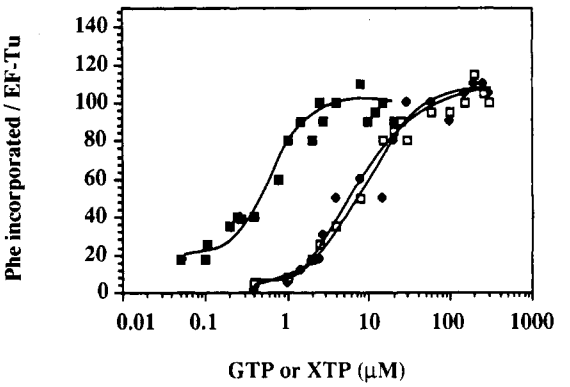

FIGURE 7: Dependence of polypeptide elongation on nucleotide concentrations. The 50-μL reaction mixtures (25 μL of mix I + 25 μL of mix II) were incubated in buffer B at the indicated nucleotide concentrations. Mix I contained 1 μM 70S ribosomes, 15 μg of poly(U), and 3 μM tRNA^{Phe}. Mix II contained 1 mM ATP, 0.05 μM EF-Tu wt or D138N, 0.2 μM EF-Ts, 8 mM [^{3}H]phenylalanine (0.1 Ci/mmol), 5 μM tRNA^{Phe}, 0.015 μM purified phenylalanyl-tRNA synthetase, and 0.1 μM EF-G. For the EF-Tu D138N/XTP system, the GTP concentration was fixed to 100 μM and the XTP concentration was varied (■) or the XTP concentration was fixed to 10 μM and the GTP concentration was varied (◆). For the EF-Tu wt-GTP system, the GTP concentration was varied as indicated (□). The elongation rate was measured by taking a sample of 30 μL after a 20-min incubation at 30 °C. The average background values subtracted correspond to those in Figure 2.

correspond to the K'_m of the coupled EF-G GTPase activity (7–15 μM). However, with this system it was not possible to discriminate between the role of the EF-Tu and EF-G factors. To further analyze the dependence of poly(phenylalanine) synthesis on the EF-Tu- and EF-G-mediated energy consumption, we performed the experiments illustrated in Figure 7. In the presence of a saturating amount of XTP and varying concentrations of GTP, the K'_m of poly(phenylalanine) synthesis was about 10 μM for both EF-Tu wt and EF-Tu D138N, a value similar to that found by Cabrer *et al.* (1976)

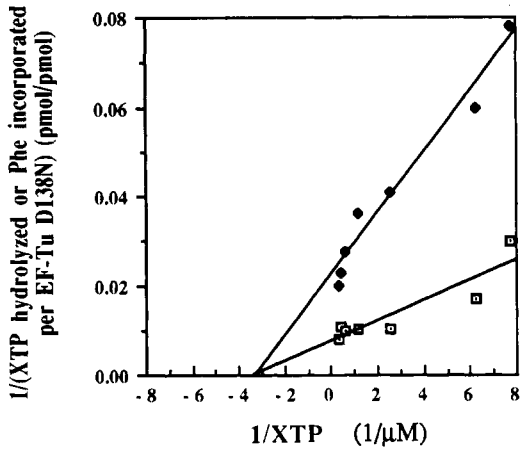

FIGURE 8: EF-Tu XTPase activity and poly(phenylalanine) synthesis show the same K_m for XTP. The 50-μL reaction mixtures (25 μL of mix I + 25 μL of mix II) were incubated in buffer B at the XTP concentrations indicated. Mix I contained 1 μM 70S ribosomes, 15 μg of poly(U), and 3 μM $tRNA^{Phe}$. Mix II contained 1 mM ATP, 100 μM GTP, 20 μM [γ-^{32}P]XTP (0.1 Ci/mmol), 0.05 μM EF-Tu D138N, 0.2 μM EF-Ts, 8 mM [^{3}H]phenylalanine (0.1 Ci/mmol), 5 μM $tRNA^{Phe}$, 0.015 μM purified phenylalanyl-tRNA synthetase, and 0.1 μM EF-G. After a 20-min incubation, 20-μL samples were withdrawn to measure the XTP hydrolysis (□) and the phenylalanine incorporation (◆). The average background values were comparable to those in Figure 5.

and Chinali and Parmeggiani (1980) for EF-Tu wt. With a saturating amount of GTP and varying concentrations of XTP, the K'_m value of EF-Tu D138N decreased to about 0.5 μM. Obviously, rate-limiting amounts of GTP reflected the activity of the EF-G-dependent GTPase, whereas when XTP was rate-limiting the EF-Tu D138N-dependent activity was measured. As shown in Figure 8, in this system the XTP K'_m of EF-Tu D138N-dependent poly(phenylalalanine) synthesis was found to be around 0.3 μM and corresponded to the XTPase activity of EF-Tu D138N.

From the work of Chinali and Parmeggiani (1980), one could deduce that the tight coupling of the elongation factor GTPase activities with the amino acid incorporation required concentrations of $MgCl_2$ lower than 9–10 mM. Higher concentrations of $MgCl_2$ drastically uncoupled the GTPase from the translational activity. To analyze separately the extent of the EF-G and EF-Tu coupling during elongation, we compared the XTPase and GTPase activities with the poly-(phenylalanine) synthesis in the EF-Tu D138N/XTP system. As shown in Figure 9A, the EF-G-dependent GTPase activity remains tightly coupled to poly(phenylalanine) synthesis in a stoichiometric 1:1 ratio up to 8 mM $MgCl_2$, whereas at higher $MgCl_2$ concentrations the decrease in phenylalanine incorporation is associated with a strong increase in the EF-G GTPase activity. Unlike the EF-G GTPase activity, the EF-Tu D138N XTPase activity decreased at higher $MgCl_2$ concentrations, following nearly the same pattern of phenylalanine incorporation (Figure 9B). Only at $MgCl_2$ concentrations higher than 12 mM did the EF-Tu D138N-dependent XTPase activity become significantly uncoupled from peptide bond formation.

DISCUSSION

In this work we have extended the characterization of EF-Tu D138N, a mutant with a modified substrate specificity, that has been used to distinguish the energy consumed by EF-Tu and EF-G during the elongation cycle. The analysis of several of its intrinsic properties and its behavior in protein biosynthesis under diverse experimental conditions show that

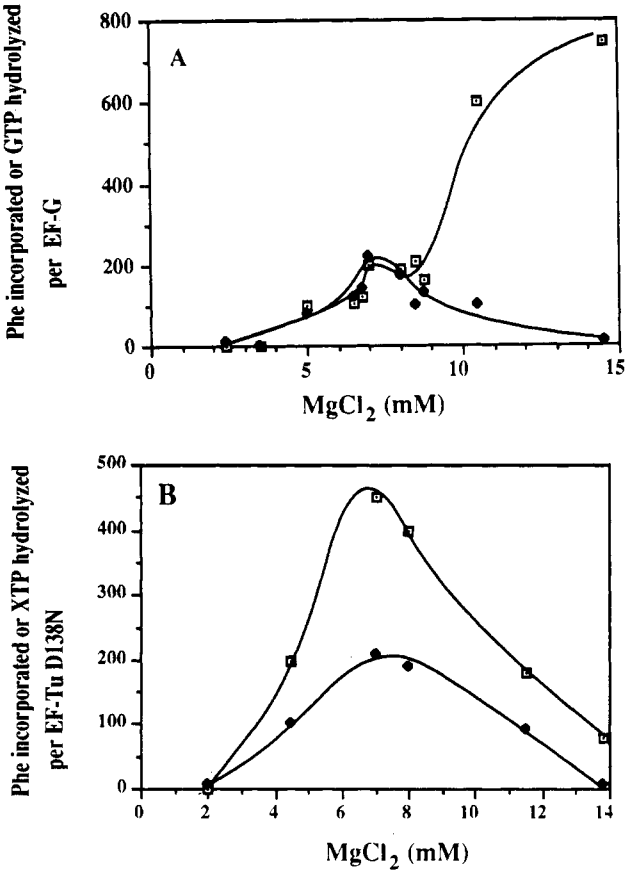

FIGURE 9: Extent of coupling of nucleotide hydrolysis and peptide bond formation for EF-G (A) and EF-Tu (B) as function of different magnesium concentrations. The 30-μL reaction mixtures (15 μL of mix I + 15 μL of mix II) were incubated at the indicated concentrations of $MgCl_2$. Mix I contained 1 μM 70S ribosomes, 15 μg of poly(U), and 3 μM $tRNA^{Phe}$ in buffer B with the $MgCl_2$ as indicated. Mix II contained 1 mM ATP, 0.05 μM EF-Tu D138N, 0.2 μM EF-Ts, 8 mM [^{3}H]phenylalanine (0.1 Ci/mmol), 5 μM $tRNA^{Phe}$, 0.015 μM purified phenylalanine-tRNA synthetase, and 0.05 μM EF-G in buffer B at the indicated $MgCl_2$ concentrations. In panel A, poly-(phenylalanine) synthesis was performed with the EF-Tu D138N-XTP system using 10 μM XTP and 20 μM [γ-^{32}P]GTP (0.1 Ci/mmol) to measure the EF-G-mediated GTP hydrolysis (□) and its ratio to phenylalanine incorporation (◆). In panel B, the EF-Tu D138N/XTP system with [γ-^{32}P]XTP (10 μM, 0.1 Ci/mmol), 20 μM GTP was used to determine the XTP hydrolysis mediated by EF-Tu D138N (□) and phenylalanine incorporation (◆). Samples of 10 μL were withdrawn after a 30-min incubation at 30 °C to measure the nucleoside triphosphate hydrolysis and the phenylalanine incorporation. The average background values subtracted, obtained with systems lacking EF-G (A) and EF-Tu (B), respectively, were 30 and 50 pmol of XTP hydrolyzed/pmol of EF-Tu D138N and 5 and 7 pmol of phenylalanine incorporated/pmol of EF-Tu D138N, respectively.

the EF-Tu D138N/XTP system closely corresponds to the EF-Tu wt/GTP system. Our results are in agreement with earlier observations of Hwang and Miller (1987), indicating that EF-Tu D138N is an active protein capable of binding xanthosine nucleotides. The differences that we observed between EF-Tu D138N and EF-Tu wt were either not significant or, as in the case of the number and length of the poly(phenylalanine) chains, did little to affect the resulting rate of poly(phenylalanine) synthesis. As a consequence, they are not relevant for the study of the basic mechanism of elongation. Our conclusion is that EF-Tu D138N/XTP is a suitable system for investigating bioenergetic aspects of the elongation process, including the proofreading of the incoming EF-Tu-bound aminoacyl-tRNA (Weijland & Parmeggiani, 1993; A. Weijland, unpublished results).

It is well established that EF-Tu can bind only one molecule of GTP and, at least in *E. coli* EF-Tu·GDP, is released from the ribosome after GTP hydrolysis. Moreover, there is strong evidence that the GDP/GTP exchange cannot take place on the ribosome-bound EF-Tu (Wolf *et al.*, 1977). This is in agreement with the observations that in a system lacking EF-Ts the stoichiometry between GTP hydrolysis and peptide bond formation is also 2 (Ehrenberg *et al.*, 1980; Bilgin *et al.*, 1992). From these observations, the most likely conclusion is that two EF-Tu·GTP (XTP) complexes participate in each elongation cycle.

EF-Tu D138N allows the precise evaluation of the extent of coupling of the EF-Tu- and EF-G-dependent nucleoside triphosphatase activities in the complete elongation system. The observation that under rate-limiting amounts of EF-Tu D138N the K'_m of the XTPase activity is comparable to the K'_m for XTP of poly(phenylalanine) synthesis proves the strict coupling between these two reactions (Chinali & Parmeggiani, 1980). This shows that the 2 XTP molecules consumed for each elongation cycle are part of the basic mechanism leading to peptide bond formation. In agreement with this is also the observation that 2 molecules of XTP are required for each elongation cycle even when the concentration of ribosomes is varied. This excludes the possibility that inactive ribosomes may influence the stoichiometry via idling reactions.

The different degree of the uncoupling of the EF-Tu and EF-G GTPase activity as a function of $MgCl_2$ concentrations during elongation became evident by using EF-Tu D138N. A 1:1 stoichiometry between EF-G-dependent GTP hydrolysis and poly(phenylalanine) incorporation took place only at magnesium ion concentrations lower than 9 mM; at concentrations higher than 9 mM, the EF-G GTPase became increasingly uncoupled from poly(phenylalanine) synthesis. Unlike this, the EF-Tu D138N XTPase starts to become uncoupled from the 2:1 stoichiometry only at concentrations of magnesium ions higher than 12 mM. The much lower degree of the EF-Tu uncoupling *vs* that of EF-G reflects the much tighter interaction between the EF-Tu·GTP·aminoacyl-tRNA complex and the mRNA·ribosome that is associated with the burstlike hydrolysis of the EF-Tu-bound GTP. In this context one should stress that at magnesium concentrations lower than 9 mM, the total number of XTP plus GTP molecules hydrolyzed for each elongation cycle was 3, as already reported (Weijland & Parmeggiani, 1993). It is also noteworthy that the K'_m (300–600 nM) of the EF-Tu dependent XTPase activity during poly(phenylalanine) synthesis lies in the same range as the K'_d for XTP of EF-Tu D138N (750 nM) in the absence of aminoacyl-tRNA.

To which purpose are the two EF-Tu·GTP complexes used in protein synthesis? No unambiguous answer is to date possible. In our opinion, the most plausible hypothesis is that both hydrolyses are engaged in the binding of aminoacyl-tRNA to the ribosomal A-site. Pre-steady-state kinetic studies have shown that the EF-Tu binding to the ribosomal A-site is a complex process (Thompson & Dix, 1982; Rodnina *et al.*, 1993; Noller, 1991), constituted by a succession of several transitional states, during which the proofreading process takes place. A sequential hydrolysis of the two EF-Tu·GTP complexes, associated with these transitional states, could play an important role in the proofreading mechanism for the selection of the correct aminoacyl-tRNA, according to the theoretical model of Hopfield (1974) and Ninio (1975). The sequential exit of the two EF-Tu·GDP complexes would increase the possibility to discard in two successive proofreading steps aminoacyl-tRNA molecules with near-cognate anticodons, of which the difference in affinity with the correct aminoacyl-tRNA is very small. In the past few years, Nierhaus and collaborators [for references see Nierhaus *et al.* (1993)] have proposed an allosteric three-site model, in which the reactivity to tRNA of the ribosomal A-site and E-site is interdependent. Occupation of the E-site by tRNA decreases the affinity of the A-site to aminoacyl-tRNA, and *vice versa* the occupation of the A-site with aminoacyl-tRNA decreases the affinity of the E-site toward discharged tRNA. Although a consensus about this model has not yet been reached (Kirillov & Semenkov, 1986; Wintermeyer *et al.*, 1990), one could speculate that one of the two EF-Tu·GTP molecules might be involved in the ejection of deacylated tRNA from the E-site, an effect that would favor the binding to the A-site of the incoming aminoacyl-tRNA. The binding to the A-site would then be completed by the hydrolysis of the second GTP molecule. It is interesting to refer to the existence of two (L7/L12) dimers, the presence of which has been reported to be required for optimal poly(phenylalanine) synthesis and EF-Tu-dependent GTPase, whereas only one dimer is needed for optimal GTPase activity dependent upon EF-G (Möller, *et al.* 1983).

Obviously, all these questions are tightly connected with the intrinsic nature of the complex between EF-Tu·GTP(XTP) and aminoacyl-tRNA. The existence of a pentameric complex in solution still lacks direct experimental evidence and has not been supported by biochemical and physicochemical observations (Bensch *et al.*, 1991; Abrahams *et al.*, 1991). Nonetheless, one should emphasize that genetic and biochemical results carried out with two mutated EF-Tu species favor the possibility of cooperative interactions between two EF-Tu molecules in solution (Vijgenboom *et al.*, 1985; Hughes *et al.*, 1987; Vijgenboom & Bosch, 1989; Anborgh *et al.*, 1991). Recently, Ehrenberg *et al.* (1993) have reported on the basis of kinetic evidence that the pentameric complex is destabilized at a temperature as low as 20 °C (Thompson & Dix, 1982). At this temperature, the active EF-Tu complex with aminoacyl-tRNA should be represented by the canonical ternary complex, despite the fact that also in this conditions 2 XTP molecules are hydrolyzed for each elongation cycle, as shown in this work. It has also been proposed (Weijland & Parmeggiani, 1993) that the formation of the pentameric complex occurs on the ribosome.

To our knowledge, in the bioenergetics of the translational apparatus, EF-Tu D138N is so far the sole protein that can hydrolyze XTP for a highly specific well-defined function. This unique property can be exploited for discriminating the various GTPase activities involved in protein biosynthesis.

Why was not the requirement for 2 GTP molecules for the EF-Tu function in elongation discovered long ago? Several circumstances may have contributed to the misinterpretation of the experimental data [see also Weijland and Parmeggiani (1994)]. The fact that EF-Tu binds only 1 molecule of GTP and forms a stable complex with aminoacyl-tRNA gave reasons for considering the ternary complex as the active multimolecular unit in protein biosynthesis. Practical problems in determining the EF-Tu stoichiometry arose from the fact that protein biosynthesis *in vitro* even in its most simplified system shows considerable experimental difficulties, such as the functional heterogeneity of the ribosomal population and the presence of a second GTPase dependent on EF-G and of other nucleotidases. The calculation of precise stoichiometries was problematic due to the uncertainty of the correction factors for background activities. All of these reasons influenced the interpretation of experimental results, favoring a 2:1 stoi-

chiometry as a logical value for the sum of the EF-Tu and EF-G-dependent GTP hydrolysis. As a typical example, the hydrolysis of 3 molecules of GTP per elongation cycle reported by Chinali and Parmeggiani (1980) was considered to be the consequence of uncoupled GTPases.

In conclusion, our results strongly suggest that the 2:1 stoichiometry is associated with the basic mechanisms of the EF-Tu-dependent amino acid incorporation. Only by using noncognate aminoacyl-tRNA it was possible to observe a clear dissociation from the 2:1 pattern (Weijland & Parmeggiani, 1993; Ruusala *et al.*, 1991; Dix & Thompson, 1986). The model based on 2 nucleoside triphosphate molecules hydrolyzed by EF-Tu for each elongation cycle opens a large number of questions that only systematic work spanning the coming years may answer.

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