Mutagenesis of Three Residues, Isoleucine-60, Threonine-61, and Aspartic Acid-80, Implicated in the GTPase Activity of *Escherichia coli* Elongation Factor Tu†

Ivo M. Krab and Andrea Parmeggiani*

Groupe de Biophysique-Equipe 2, Ecole Polytechnique, F-91128 Palaiseau, France

Received April 28, 1999; Revised Manuscript Received July 20, 1999

ABSTRACT: The properties of variants of elongation factor (EF) Tu mutated at three positions implicated in its GTPase activity are presented. Mutation I60A, which reduces one wing of a "hydrophobic barrier" screening off the nucleophilic water molecule found at the GTP γ-phosphate, causes a reduction of the intrinsic GTPase activity contrary to prediction and has practically no influence on other properties. Mutation D80N, which in the isolated G-domain of EF-Tu caused a strong stimulation of the intrinsic GTPase, reduces this activity in the intact molecule. However, whereas for wild-type EF-Tu complex formation with aa-tRNA reduces the GTPase, EF-Tu[D80N] shows a strongly increased activity when bound to Phe-tRNA. Moreover, ribosomes or kirromycin can stimulate its GTPase up to the same level as for wild-type. This indicates that a local destabilization of the magnesium binding network does not per se cause an increased GTPase but does affect its tight regulation. Interestingly, mutant D80N sequestrates EF-Ts by formation of a more stable complex. Substitutions T61A and T61N induce low intrinsic GTPase, and the stimulation by ribosome is less for T61A than for T61N but still detectable, while kirromycin stimulates the GTPase of both mutants equally. This provides more evidence that stimulation by kirromycin and ribosomes follows a different mechanism. The functional implications of these mutations are discussed in the context of a transition state mechanism for catalysis. An alternative structural explanation for the strong conservation of Ile-60 is proposed.

Elongation factor (EF)[1] Tu is a guanine nucleotide-binding protein that takes care of the efficient transport of aminoacylated tRNAs to the A-site of the ribosome in the elongation cycle of protein biosynthesis (see ref *1*). It moreover plays an active role in the selection of the correct aa-tRNA from the about 40 different species available, ensuring thus a high accuracy of the process. EF-Tu has specific interactions with many different partners in its functional cycle and shows remarkable structural dynamics. In the rather compact GTP form it has high affinity for aa-tRNA. The EF-Tu·GTP·aa-tRNA complex can interact with the A-site of the mRNA-programmed ribosome; in the case of correct codon−anticodon interaction this elicits a fast one-round GTP hydrolysis. The resulting GDP form has a much more open triangular structure, in which certain residues have moved through as much as 40 Å compared to the GTP form (*2*−*5*), and has low affinity for aa-tRNA, thus leaving the ribosome and allowing a new peptide bond to be formed. The precise interactions changing EF-Tu from a rather inefficient GTP hydrolase into a very good one have not yet been determined. To obtain the maximum stimulation, a correct codon−anticodon interaction on the ribosome is known to be an essential requirement (*6*−*8*), although partial stimulation can be obtained by ribosomes alone (*9, 10*), by high monovalent cation concentrations (*11, 12*), and by the antibiotic kirromycin alone (*13*) or even more strongly in the complex with aa-tRNA (*6*). Thus an intricate interplay seems to be necessary to obtain the optimal catalytic configuration. For the possible involvement of a residue from a ribosomal protein or a base from rRNA, analogous to the situation for the small guanine nucleotide-binding protein family (*14*), no concrete evidence exists (*1*). Research into the residues from EF-Tu itself involved in catalysis has not been able to unequivocally attribute a catalytic role to any specific one, thus favoring a transition state stabilization mechanism for hydrolysis (*1, 15, 16*).

Among the residues conserved in the elongation factors are those involved in nucleotide binding [four groups of residues forming the guanine nucleotide-binding protein "fingerprint" (*17, 18*)] and also others that are characteristic for this guanine nucleotide-binding protein family but whose function is not clear. In the first group are Thr-61 and Asp-80, two residues having a direct and a water-mediated interaction with the essential magnesium complexed to the nucleotide, respectively. In the second group is Ile-60, a residue that was found in the 3D GTP structure to form a "hydrophobic barrier", together with Val-20 located opposite to it in the P-loop. This "barrier" would screen off the water molecule located at the γ-phosphate in position for a nucleophilic attack. In this context, His-84 is considered the general base activating this water molecule, which supposes that the "gate" opens periodically by thermal movement, allowing the catalysis by His-84 to take place.

† This research was carried out in the framework of Contract ERB-CHRXCT 940510 (Program Human Capital and Mobility of the E.U.) and was supported by grants from the Ligue Nationale Française Contre le Cancer, Fédération Nationale des Centres de Lutte Contre le Cancer, and Association pour la Recherche sur le Cancer (No. 6377).

* Corresponding author: telephone, (+33) 1 69 33 41 80; fax, (+33) 1 69 33 48 40; e-mail, andrea@poly.polytechnique.fr.

[1] Abbreviations: EF, elongation factor; ME, 2-mercaptoethanol.

10.1021/bi9909748 CCC: $18.00
Published on Web 09/17/1999

In this work we set out to define more precisely the role of the magnesium ion and the hydrophobic barrier in the GTPase reaction by characterizing mutants of these three residues. In the isolated domain 1 (G-domain) the substitution Asp-80→Asn was found to cause a strong increase in the intrinsic GTPase rate, concomitant with a structural destabilization (*19*). This effect suggested a destabilization of the magnesium binding network as an intermediary step of the transition leading to the hydrolysis of GTP. We extended that work here by examining the same mutation in the intact EF-Tu. Thr-61 was suggested to be involved in transmitting the ribosome signal for triggering the GTPase on the basis of mutations of the homologous residue in *Thermus thermophilus* EF-Tu (*20*). We present an in-depth analysis of mutants of Thr-61 to Ala and Asn, in which differences between the *T. thermophilus* and *Escherichia coli* situations are revealed. To evaluate the proposed role of the "hydrophobic gate" residue Ile-60 in reducing the intrinsic GTPase, we characterized the mutation Ile-60→Ala, which complements previous work describing the properties of Val20→Gly (*21*).

EXPERIMENTAL PROCEDURES

Biological Materials. Ribosomes, EF-G, Phe-tRNA^{Phe} synthetase, and partially purified tRNA^{Phe} (5−70%) were prepared from *E. coli* as reported (*10, 13*). In the case of tRNA^{Phe} also purified preparations obtained from Sigma were used.

Construction, Overproduction, and Purification of EF-Tu Mutants. For rapid purification purposes wt EF-Tu and mutants were overproduced as fusion with glutathione *S*-transferase and purified as described before (*22*). Mutations were introduced by the "Unique Site Elimination" method (*23*), using a kit from Pharmacia. As a substrate for the mutagenesis reactions we used pGtAM, a derivative of plasmid pGEX-2T*tufA* (*24*), which codes for a thrombin-cleavable glutathione *S*-transferase-fused *E. coli* EF-Tu (*tufA*). In pGtAM the *Nco*I site from pGEX-2T*tufA* has been eliminated, and the unique *Nru*I site in the *tufA* sequence—which turned out to digest poorly—has been modified by a silent mutation generating a unique overlapping *Nco*I site, used for the selection procedure. The mutagenic oligonucleotides for the amino acid substitutions were 5′-CT CGT GGT GCC ACC ATC AA -3′ for I60A, 5′-GT GGT ATC GCC ATC AAC-3′ for T61A, 5′-CGT GGT ATC AAC ATC AAC AC-3′ for T61N, and 5′-CAC GTA AAC TGC CCG-3′ for D80N (modified bases underlined). Because transformation by the calcium chloride method advised in the kit proved not efficient enough, the "simple and efficient method" of Inoue was used instead (*25*). Selected clones were verified by ds-DNA sequencing of the plasmid, and a mutagenesis efficiency of more than 50% was found. The concentration of the purified proteins was determined by the Bio-Rad protein assay, using bovine serum albumin as standard.

Functional Assays. Poly(Phe) synthesis was determined kinetically from the formation of hot trichloroacetic acid-insoluble material (*26*) and the GTPase activity from liberation of [^{32}P]P_i using the molybdate method (*27*). Nucleotide dissociation rates and affinities (*11, 15, 28*) and protection of aa-tRNA by EF-Tu against spontaneous hydrolysis (*29*) were measured as described. Detailed reaction conditions are reported in the legends to the figures.

Table 1: Nucleotide Interaction Parameters[a]

	GDP			GTP		
	$k_{off} \times 10^4$ (s^{-1})	$k_{on} \times 10^{-4}$ [b] ($s^{-1}\,M^{-1}$)	K_d (μM)	$k_{off} \times 10^4$ (s^{-1})	$k_{on} \times 10^{-4}$ [b] ($s^{-1}\,M^{-1}$)	K_d (μM)
wt	2.2	34	0.00064	79	2.3	0.34
I60A	2.1	34	0.00062	163	1.5	1.1
T61A	2.6	43	0.00061	165	4.1	0.40
T61N	2.3	38	0.00060	187	2.9	0.64
D80N	260	1.9	1.4	128	∼0.3[c]	∼5[c]

[a] Shown are apparent rate constants for dissociation (k_{off}) and association (k_{on}) of GDP and GTP and the equilibrium dissociation constants (K_d) measured at 0 °C. [b] Calculated as $k_{on} = k_{off}/K_d$ assuming a two-state association/dissociation reaction without intermediate steps. [c] Value estimated from nucleotide binding versus nucleotide concentration.

RESULTS

Production, Expression, and Purification of Mutant EF-Tu's. EF-Tu wt and mutants I60A, T61A, and T61N are overexpressed to a high level and show good solubility (>70%), giving yields of up to 2 mg of EF-Tu (unfused)/g of cells (wet weight). Mutant D80N, on the other hand, is well expressed, but less of the protein is found in the S30 supernatant fraction. This mutant also shows a high stability of the complex with EF-Ts, making an ultimate Superdex 75 gel filtration step necessary to separate free protein from the EF-Tu[D80N]·EF-Ts complex in order to obtain an EF-Ts-free preparation. These observations agree with our recent findings (unpublished) that several EF-Tu mutants with lower nucleotide affinity have problems to fold correctly in the cell. It does not indicate any structural instability, however. No precipitation phenomena or other stability problems were observed with the purified factors.

Interaction with GDP and GTP. Concerning the dissociation constants and the dissociation rate constants, we found that for the GDP interaction the mutants of the effector region residues 60 and 61 have parameters that are not far from those of wt EF-Tu (Table 1). For the GTP interaction we see an increase in the dissociation rates by a factor of about 2, whereas the affinity is lower by about a factor 3 for EF-Tu[I60A] and by a factor 2 for EF-Tu[T61N].

The mutant of residue 80, on the other hand, shows a dramatic alteration. The GDP affinity is 3 orders of magnitude lower, the dissociation rate being 2 orders of magnitude higher. As a consequence, the deduced association rate is also more than 1 order of magnitude lower. For GTP the changes are less dramatic. The affinity is again lower than for wt, but the difference is only 1 order of magnitude and the dissociation rate is less increased than for the other mutants.

Activity in Poly(Phe) Synthesis. To evaluate the overall effect of the mutations on EF-Tu's ability to sustain its physiological function, we performed poly(U)-directed poly(Phe) synthesis (Figure 1) at a magnesium concentration of 10 mM to ensure that the differences seen are not the result of insufficient saturation of EF-Tu·GTP with aa-tRNA (see below). We observed that EF-Tu[I60A] can sustain the same rate as wt and that EF-Tu[T61N] is little affected, showing 80% of the wt rate. On the other hand, EF-Tu[T61A] and EF-Tu[D80N] are less efficient, at 28% and

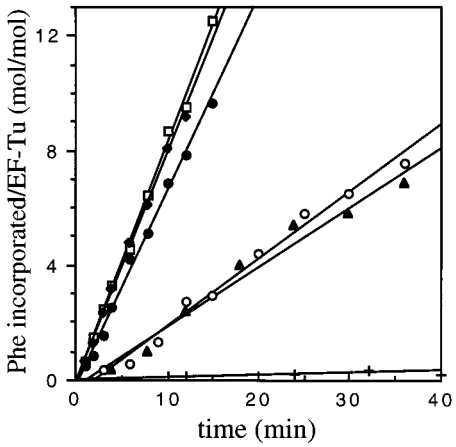

FIGURE 1: Poly(U)-directed poly(Phe) synthesis by EF-Tu wt (□), EF-Tu[I60A] (◆), EF-Tu[T61A] (○), EF-Tu[T61N] (●), and EF-Tu[D80N] (▲) or in the absence of EF-Tu (+). The final reaction mixture (60 μL) contained 50 mM Hepes−KOH, pH 7.5, 70 mM NH_4Cl, 10 mM $MgCl_2$, and 7 mM ME (buffer A) with 0.9 mM ATP, 1.0 mM GTP, 3.8 mM phosphoenolpyruvate, 30 μg/mL pyruvate kinase (EC 2.7.1.40), 2 units/mL myokinase (EC 2.7.4.3), 50 nM EF-Ts, 0.8 μM EF-G, 20 μM [^{14}C]Phe (specific activity 147 dpm/pmol), 3 μM $tRNA^{Phe}$, 26 nM phenylalanine-tRNA synthetase (EC 6.1.1.20), 1.5 μM ribosomes with $tRNA^{Phe}$ preloaded in the P-site, 100 μg/mL poly(U), and 0.5 μM EF-Tu as indicated. The reaction was started by adding together a mix containing ribosomes, poly(U), and $tRNA^{Phe}$ in buffer A and a mix containing all other factors and components which had been separately incubated at 30 °C for 15 min. Incubation was continued at 30 °C, 6 μL samples were withdrawn at indicated times and spotted on glass fiber filters, and hot trichloroacetic acid-insoluble radioactivity was determined.

25%, respectively. They are thus almost equally affected in their physiological function, despite the quite different effects of the mutation on the nucleotide interaction. The two different mutations of Thr-61 behave also strikingly differently, pointing to the importance of the Thr-61-OH→Mg^{2+} bond for the dynamic cycle.

Enzymatic Binding of aa-tRNA to Programmed Ribosomes. To analyze the effect of the mutations on the Mg^{2+} requirement for function, we examined the extent of enzymatic binding of Phe-$tRNA^{Phe}$ to the A-site of programmed ribosomes as a function of the Mg^{2+} concentration (Figure 2). EF-Tu[I60A] is indistinguishable from wt, as could be expected from the absence of an interaction with the magnesium. EF-Tu[T61N] and EF-Tu[D80N] both reach 50% of the maximum binding at 1.5−2 mM higher [Mg^{2+}], whereas EF-Tu[T61A] needs about 2.5 mM more than wt to reach this level. These results show a close qualitative agreement with the expected ability of the substituting residue to still complex the magnesium ion. They also show that the residue at position 61 does not not really need to have an interaction with the magnesium ion for EF-Tu to be able to sustain this partial reaction efficiently, whereas in the complete elongation cycle loss of this contact leads to a much reduced activity.

Ester Bond Protection of aa-tRNA. Whereas the ability to participate in poly(Phe) synthesis and enzymatic binding, shown in the previous experiments, establishes that the mutants have conserved the ability to productively interact with aa-tRNA, we also tested whether the property of EF-Tu to protect the ester bond of aa-tRNA from spontaneous hydrolysis has been altered by the mutations. As shown in

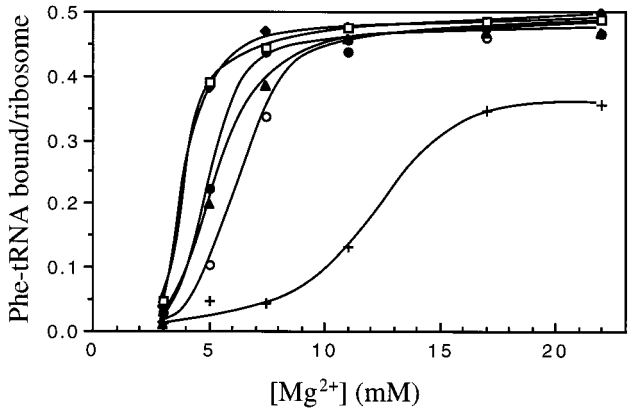

FIGURE 2: Enzymatic binding of [^{14}C]Phe-$tRNA^{Phe}$ to the ribosomal A site as function of [Mg^{2+}] by EF-Tu wt (□), EF-Tu[I60A] (◆), EF-Tu[T61A] (○), EF-Tu[T61N] (●), and EF-Tu[D80N] (▲) or in the absence of EF-Tu (+). The reaction was started by adding together a mix containing preformed EF-Tu·GTP·[^{14}C]Phe-$tRNA^{Phe}$ ternary complexes (mix I) and a mix containing poly(U)-programmed ribosomes with $tRNA^{Phe}$ preloaded in the A-site (mix II). After 5 min at 30 °C the reaction (50 μL) was filtered through nitrocellulose. Filters were immediately washed twice with 2 mL of ice-cold buffer, and the retained radioactivity was counted. Mix I (42 μL) consisted of 8 μL of a preincubated $tRNA^{Phe}$ charging reaction and other components to give a final composition of 50 mM Hepes−KOH, pH 7.5, 70 mM NH_4Cl, $MgCl_2$ at variable concentrations, 14 mM ME, 1.2 mM ATP, 0.24 mM GTP, 2.4 mM phosphoenolpyruvate, 30 μg/mL pyruvate kinase, [^{14}C]Phe-$tRNA^{Phe}$ [formed from 0.9 μM $tRNA^{Phe}$ and 1.0 μM [^{14}C]Phe (specific activity 1105 dpm/pmol) by 8 nM phenylalanine-tRNA synthetase], and 0.54 μM each EF-Tu. Mix II (8 μL) contained 50 mM Hepes−KOH, pH 7.5, 70 mM NH_4Cl, 8 mM $MgCl_2$, 14 mM ME, 500 μg/mL poly(U), 1.7 μM $tRNA^{Phe}$, and 1.3 μM ribosomes. Both mixes were incubated separately at 30 °C for 15 min before the reaction was started.

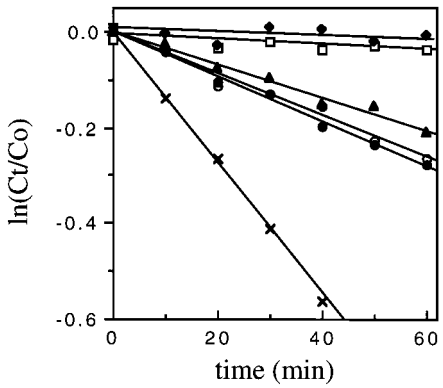

FIGURE 3: Protection against spontaneous hydrolysis of the ester bond of Phe-$tRNA^{Phe}$ by EF-Tu wt (□), EF-Tu[I60A] (◆), EF-Tu[T61A] (○), EF-Tu[T61N] (●), and EF-Tu[D80N] (▲) or in the absence of EF-Tu (+). The final reaction mixture (62 μL) contained 25 mM Hepes−KOH, pH 7.5, 60 mM NH_4Cl, 10 mM $MgCl_2$, 7 mM ME, 0.5 mM GTP, 1 mM phosphoenolpyruvate, 30 μg/mL pyruvate kinase, 1.5 μM [^{14}C]Phe-$tRNA^{Phe}$ (specific activity 331 dpm/pmol), and 3 μM EF-Tu as indicated. The reaction (at 30 °C) was started by adding 15 μL of buffer containing [^{14}C]Phe-$tRNA^{Phe}$ to 47 μL of mix with the other components which had been preincubated at 30 °C for 15 min to convert EF-Tu·GDP to EF-Tu·GTP. At indicated times 8 μL samples were taken and spotted onto glass fiber filters, and cold trichloroacetic acid-insoluble material on the filters was measured.

Figure 3, at 10 mM Mg^{2+} marked differences become evident. In fact, whereas the behavior of EF-Tu[I60A] is again undistinguishable from wt, both mutations of the adjacent Thr-61 are equally less effective: the half-life of

Table 2: GTPase Activities[a]

	intrinsic $k_{app} \times 10^5$ (s^{-1})	+Phe-RNA^{Phe} $k_{app} \times 10^5$ (s^{-1})	+ribosomes $k_{app} \times 10^5$ (s^{-1})
wt	8.0	1.6	334
I60A	5.2	2.9	178
T61A	1.4	2.0	8.8
T61N	1.6	2.4	50.9
D80N	3.7	19.2	326

[a] Apparent rate constants of the liberation of [^{32}P]phosphate from [γ-^{32}P]GTP at 30 °C. Final reaction conditions were 50 mM Hepes−KOH, pH 7.5, 100 mM NH_4Cl, 10 mM $MgCl_2$, 7 mM ME, 1−2 mM ATP, 100 μM [γ-^{32}P]GTP (specific activity 1500 cpm/pmol), 1 mM phosphoenolpyruvate, 10 μg/mL pyruvate kinase, and 3−4 μM each EF-Tu. EF-Tu·GTP was preformed by incubating this mix without the [γ-^{32}P]GTP for 15 min at 30 °C. Reactions were started after further incubation with [γ-^{32}P]GTP on ice for 15 min by adding the stimulatory component and shifting the reaction to 30 °C. Samples were taken at indicated times and quenched in 1 M $HClO_4$/3 mM KH_2PO_4, and free [^{32}P]phosphate was determined by the molybdate/isopropyl acetate method (*10*). The background hydrolysis by ribosomes subtracted in the third column was 15.9 s^{-1}.

aa-tRNA goes down from >10 h to 90 min. Mutant EF-Tu[D80N] protects slightly better than the Thr-61 mutants. Since the affinities for aa-tRNAs are in the nanomolar range (*30*, *31*), at the micromolar concentrations used all mutant EF-Tu should at least be near-saturated with aa-tRNA. Thus, the reduced aa ester bond protection for both Thr-61 mutants and for EF-Tu[D80N] points to an anomalous interaction of the aminoacyl end of aa-tRNA with EF-Tu, whereas the orientation of the other EF-Tu domains to aa-tRNA seems to be little or not affected.

Intrinsic GTPase Activity. Effect of aa-tRNA, Ribosomes, and Kirromycin. Our main interest in characterizing these mutant EF-Tu's concerned the effects on the GTPase, both intrinsic and in interaction with EF-Tu's physiological partners. Table 2 summarizes the activities measured starting from EF-Tu·[γ-^{32}P]GTP, at high [γ-^{32}P]GTP concentration (100 μM), which should ensure near-complete saturation even for EF-Tu[D80N], so that the measured rates correspond closely to V_{max}. The wild-type shows the usual reduction in the complex with Phe-tRNA, by a factor of 5 in these circumstances. Empty ribosomes stimulate the GTPase 42 times. For the substitution I60A, the hydrophobic barrier hypothesis predicts an increased intrinsic GTPase rate. On the contrary, we observe a decrease by 35%. Complexation with Phe-tRNA^{Phe} further reduces the GTPase, although it stabilizes this mutant less than wt. Ribosomes stimulate this mutant quite well, though at 34 times also somewhat less efficiently than wt. Both mutants of the neighboring residue Thr-61 show a ca. 5-fold reduced intrinsic GTPase, and in contrast to wt, the presence of Phe-tRNA^{Phe} slightly stimulates their activity. For these mutants the ability of ribosomes to stimulate the GTPase varies with the substitution. Whereas the EF-Tu[T61N] is stimulated by ribosomes 32 times, T61A's rate is enhanced only six times. The most surprising behavior is shown by EF-Tu[D80N], whose intrinsic activity is decreased to about half. However, in the presence of Phe-tRNA^{Phe} the GTPase rate is increased to about 2.5 times the wt intrinsic rate, which corresponds to a 5-fold stimulation compared to this mutant's intrinsic catalytic activity. We furthermore observe that EF-Tu[D80N] is stimulated to the same rate as wt in the presence of ribosomes, i.e., by a factor of 88.

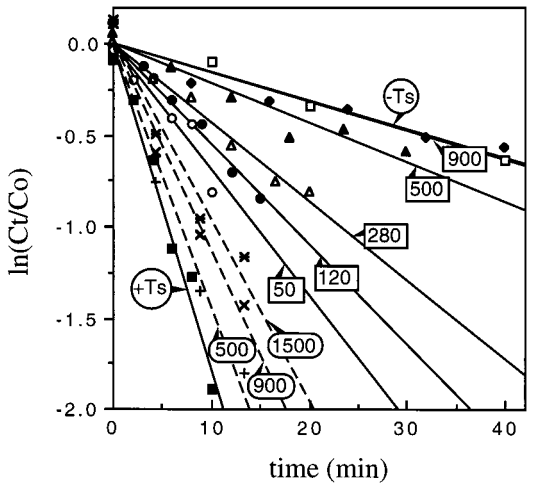

FIGURE 4: Inhibition of EF-Ts stimulation of EF-Tu[wt]·[^{3}H]GDP dissociation by increasing concentrations of external EF-Tu[wt]·GDP (dotted lines and oval labels) or EF-Tu[D80N]·GDP (solid lines and square labels). Numbers in the labels indicate the concentration of competing EF-Tu (in nM) in the reaction for the corresponding curve. The curves labeled +Ts and −Ts are the control reactions without competitor in the presence and absence, respectively, of EF-Ts. Reaction conditions were 50 mM Hepes−KOH, pH 7.5, 60 mM NH_4Cl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 30 nM EF-Tu[wt]·[^{3}H]GDP (specific activity 6300 dpm/pmol), 9 nM EF-Ts, 20 μM GDP, and indicated concentrations of various competitor EF-Tu·GDP. The reaction was started by adding a mix containing EF-Ts, competitor EF-Tu, and excess cold GDP to the preformed EF-Tu[wt]·[^{3}H]GDP on ice. Samples were filtered through nitrocellulose filters as for Figure 2.

To verify that the surprising decreased GTPase for EF-Tu[I60A] is due to a decrease in the catalytic efficiency and not some other effect, we determined both K_M and k_{cat} from a Lineweaver−Burk plot (not shown). The k_{cat} is decreased from 7.1×10^{-5} s^{-1} in the wt to 4.1×10^{-5} s^{-1} in EF-Tu[I60A], whereas the K_M is slightly increased from 0.26 to 0.44 μM. Thus the catalytic efficiency k_{cat}/K_M is reduced from 270 to 93 M^{-1} s^{-1} for this mutant.

Concerning the GTPase in the presence of the antibiotic kirromycin, we observe for EF-Tu[D80N] the same stimulated rate as for wt, similar to the activation by ribosomes; for both mutants of Thr-61 we see a moderate enhancement of about 5-fold, whereas EF-Tu[I60A] is stimulated comparably to wild type (results not shown).

Dominant-Negative-like Properties of the Mutants. We recently reported that EF-Tu[T25A] shows a behavior similar to that of so-called dominant-negative mutants of Ras proteins, i.e., mutants that inhibit native Ras in the cell by sequestration of the exchange factors that regenerate the active form of ras. Such mutations causing increased stability of the complex with the exchange factor occur in Ras proteins not only in position 17 (the equivalent of EF-Tu Thr-25) but also in position 57, the equivalent of EF-Tu Asp-80 (*32*). Interestingly, during purification of EF-Tu[D80N] we again observed tight complex formation with EF-Ts, as for EF-Tu[T25A]. Therefore, we tested whether EF-Tu[D80N] could also inhibit the stimulation by EF-Ts of [^{3}H]GDP exchange on wt EF-Tu. As shown in Figure 4, the presence of increasing concentrations of mutant strongly inhibits the function of EF-Ts. The other mutants do not show this effect (not shown). The observed differences in behavior between EF-Tu[T25A] and a double mutant EF-Tu[H22Y/T25S] had

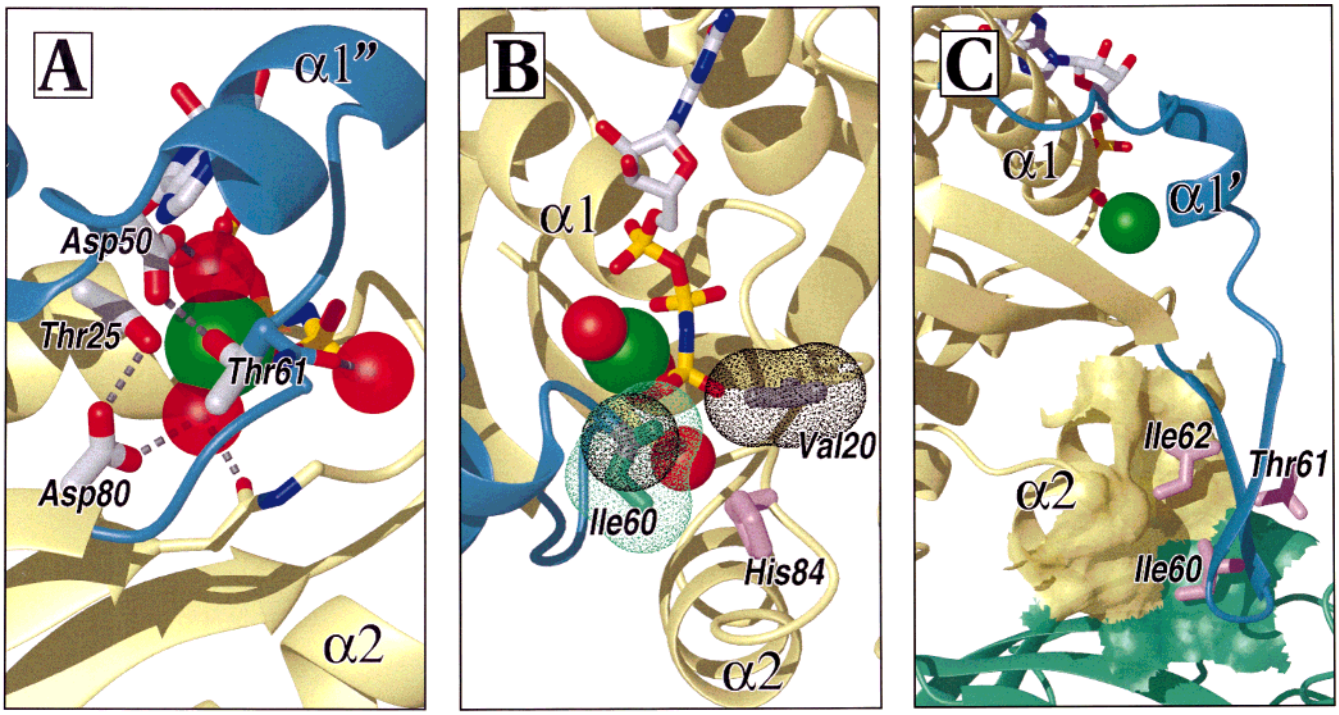

FIGURE 5: Details of structural elements discussed in the text. Residue numbers refer to *E. coli* even if the structure is from *T. thermophilus*. Panel A: Detailed view of the residues surrounding the Mg^{2+} ion (green ball) in the EF-Tu_{Tt}·GTP structure (*2*) and their interactions with each other and the water molecules (red transparent balls). Dotted lines indicate hydrogen bonds (distances 2.6−2.8 Å). The effector region (switch 1) is colored blue; the nucleotide is in part covered by the magnesium ion, water molecules, and helix α1″ from the effector region. Panel B: Close-up of the hydrophobic barrier (EF-Tu_{Tt}·GTP), in the wt situation and with an Ala statically modeled in place of Ile-60. His-84 (purple) is prevented from approaching the water molecule at the γ-phosphate by residues Val-20 and Ile-60, while an Ala at position 60 leaves more space for the barrier to open through thermal motion, assuming no dramatic changes of the local conformation. van der Waals envelopes of these residues are indicated in black dots for the modeled situation in the mutant, while the wt Ile-60 surface is dotted in green. Panel C: Overview of the structure of the switch 1 region (blue) in EF-Tu_{Ec}·GDP (*4*) and the hydrophobic contacts of residues Ile-60 and Ile-62 (purple) with the interface of helix α2 and domain 3 (green), indicated as a partially transparent solvent-accessible contact surface below the switch 1 β-hairpin. Ile-62 is in a hydrophobic pocket formed by Ala-85, Lys-89, and Asn-90 (not shown), whereas Ile-60 is surrounded by Lys-89, Thr-93, Tyr-309, and Ala-385 (not shown). Thr-61 located between the isoleucines points into the solvent, far away from the magnesium ion (green ball). The figure was prepared using MOLMOL (*41*) and POV-ray (http://www.povray.org).

led us to conclude that this phenomenon is related to the ability of EF-Tu to have a stable interaction with the magnesium ion from position 25 and not simply to the strong decrease in nucleotide affinity observed for these two mutants. Thus, we may assume that also for EF-Tu[D80N] the changed nature of the interaction with the Mg^{2+}·nucleotide is responsible for the increased stability of the EF-Tu·EF-Ts complex. This once again confirms the importance of the magnesium ion in the pathway for nucleotide ejection by EF-Ts.

DISCUSSION

This work analyzes the effect of mutations of three conserved residues of EF-Tu from *E. coli*, two of which (Thr-61 and Asp-80) are components of the magnesium binding network (illustrated in Figure 5A) and one (Ile-60) is a close neighbor to a water molecule located at the γ-phosphate of GTP (Figure 5B). Residues Ile-60 and Thr-61 are part of the switch I region of EF-Tu. In the GTP form they flank at the C-terminal side the short α helix α1″, which in the GDP form unwinds to a β-hairpin bridging helix α2 and domain 3 (*4*, *5*) (Figure 5C). The third residue examined, Asp-80, is strictly conserved in the DXXG motif just preceding the switch II region of guanine nucleotide-binding proteins. Residues Thr-61 and Asp-80 interact directly and via a water molecule, respectively, to the nucleotide-bound magnesium ion which is essential for EF-Tu's function (*33*). All three residues were implicated in the GTPase reaction in various ways in the literature. Most notably, substitution D80N increased the intrinsic GTPase in the isolated domain 1 of EF-Tu by up to 2 orders of magnitude, although the protein was strongly destabilized and high concentrations of glycerol were necessary to preserve its activity (*19*). Upon substitution of the homologue of Thr-61 to Ala, *T. thermophilus* EF-Tu was reported to have very low intrinsic GTPase activity, which could not be stimulated by ribosomes; thus this residue was suggested to play a role in transmitting the "ribosome signal" for triggering the GTPase (*20*). Ile-60, finally, was found in the GMPPNP crystal structure to be part of a hydrophobic barrier between the putative nucleophilic water located at the γ-phosphate of the nucleotide and residue His-84, which had been proposed to act as a general base in the catalysis of GTP hydrolysis (*2*). Thus, it was predicted that removing or reducing this barrier would facilitate acces of the outward-pointing His-84 side chain into the active site and therefore increase the GTPase activity of the protein.

Concerning the effect on the nucleotide interaction, the mutants can be divided into two groups: mutations of positions 60 and 61 do not influence the interaction with GDP and very modestly affect the interaction with GTP; most notably they show a slightly faster GTP dissociation. Since EF-Tu[I60A] has no direct contacts with either nucleotide

or magnesium, a local conformation disturbance seems the most likely explanation for its higher GTP dissociation rate. The similar and remarkably modest effect of the Thr-61→Ala mutation might seem to be in contrast with the prominence of the Thr-61−Mg^{2+} ion link in the crystal structures, also when compared to the drastic change caused by mutation of the opposite Thr-25 to alanine (*22*). However, this finding agrees with experimental evidence that in H-ras p21 the coordination of the homologous residue Thr-35 to magnesium is rather weak (*34, 35*), despite the presence of a similarly prominent interaction in the crystal. In EF-Tu the coordination may be even weaker, for the ras mutation T35A shows an approximately 6-fold decreased affinity for GTP, whereas the affinity of EF-Tu[T61A] practically equals that of wt. Noteworthy, the influence on the dissociation rates shown here is opposite to that found for $EF\text{-}Tu_{Tt}$: there, substitutions T62A and T62S increased the GDP dissociation rates but did not affect the GTP dissociation (*20*).

On the other hand, the substitution Asp-80→Asn shows a dramatic change of all interaction parameters. Most notably, the GDP affinity lies in the micromolar range, i.e., close to that for GTP. Thus, it mostly loses the several orders of magnitude higher affinity for GDP, typical for *E. coli* EF-Tu. Aspartate has a pK_a of 4.4 and is thus mostly ionized, making it an exclusive hydrogen bond acceptor. In the crystal structures Asp-80 is seen to accept hydrogen bonds from Thr-25 and from one water coordinated to the magnesium, which donates its other H toward the Cys-82 mc CO group. An asparagine side chain is poorly ionizable and can act both as a hydrogen bond acceptor and as a donor. Whereas a similar configuration as for Asp-80 is possible with the Asn amide donating an H to the Mg^{2+}-bound water, leaving one amide and one water H unbonded, the lack of a negative charge attracting the magnesium likely makes the bonding weaker.

Concerning the effects on the GTPase, EF-Tu[I60A] is shown here to have a decreased intrinsic GTP hydrolysis, in contrast to the prediction that reducing the hydrophobic barrier should increase the intrinsic catalysis rate. This finding is similar to that obtained several years ago with EF-Tu[V20G], removing the other wing of the hydrophobic gate, which displayed a reduced GTPase (4−5-fold) (*21*). Differently, however, whereas ribosomes hardly stimulated the EF-Tu[V20G] GTPase, EF-Tu[I60A] is stimulated to a similar extent as wt, as is the case for kirromycin. Thus, the modification seems to affect the stimulated GTPase in a similar way as the intrinsic reaction, suggesting that the effect is unspecific and that the residue is not directly involved in the mechanism of catalysis. Also mutagenesis of the proposed catalytic residue His-84 had shed doubt on its involvement in water activation, at least for the intrinsic reaction (*15, 16, 36*), and comparison with the various recent crystal structures of Ras(-like) proteins with their respective GTPase-activating proteins in a transition state-mimicking complex (*37*−*39*) also raises questions considering the similarity of the GTPase stimulation in the two systems (*1*). An important still unsolved question remains then, why Ile-60 is so highly conserved in elongation factors (conservatively substituted to Val in most archea). A possible answer may be found in the 3D structure of the GDP form of EF-Tu (Figure 5C): The hairpin loop resulting from the unwinding of helix α1″ forms a bridge over helix α2 and domain 3. Both Ile-60 and the universally conserved Ile-62 are located in hydrophobic pockets in the interface between the loop and the other two elements, whereas Thr-61 between these two residues points into the solvent. Thus these two highly conserved isoleucines may play a defined role in stabilizing the interaction of the switch II region with the domain 1−domain 3 interface in the GDP complex. Interestingly, also in EF-G the homologous residues are conserved in the highly similar α1″ part of the sequence. This makes it attractive to speculate that this structure, invisible in the EF-G·GDP crystal structure (*40*), might also feature the helix-to-loop unrolling in the GDP to GTP transition.

The surprising increase in the intrinsic GTPase that was observed for mutation D80N in the isolated G-domain (*19*) is not found for the same substitution in the intact molecule, even in the presence of glycerol (not shown). Nevertheless, the reduction of the intrinsic catalysis is changed into a 5-fold stimulation on formation of a complex with aa-tRNA. This is in marked contrast with the wild-type situation, where normally binding aa-tRNA further reduces the GTPase, thus suppressing energy loss through improductive GTP hydrolysis. Moreover, empty ribosomes stimulate the mutant's GTPase to the same turnover rate as wt, as does kirromycin. This suggests that the overall efficiency of the (physiological) stimulated GTPase may not be negatively affected. However, the increased rate seen with aa-tRNA suggests that the mutation causes a conformational modification which makes the regulation of the GTPase less tight. In the absence of the constraints exercised by domains 2 and 3 on domain 1, this also leads to an increased intrinsic activity. The important influence of domains 2 and 3 on the conformation and regulation of domain 1 is also emphasized by the observation that the extreme lability that the G-domain[D80N] displayed is absent here.

Whereas the two substitutions of Thr-61 both impair the intrinsic GTPase, the complexation with aa-tRNA causes a small increase, though much less than for EF-Tu[D80N] and remaining below the wt intrinsic rate. The catalysis of both mutants is comparably enhanced by kirromycin, but ribosomes stimulate EF-Tu[T61A] much less than EF-Tu[T61N], which is activated to an extent approaching that of wt. This provides more evidence that GTPase activation by kirromycin and ribosomes follows different mechanisms (*22*). The low ribosome stimulation of the EF-Tu[T61A] GTPase we find here was not observed for *T. thermophilus* $EF\text{-}Tu_{Tt}$[T62A] (*20*) at an assay temperature of 37 °C that is suboptimal for the thermophilic enzyme. The fact that even an Asn at this position—which may weakly bind the Mg^{2+} provided a conformational change to accommodate the longer side chain takes place—shows better activation by the ribosome indicates that for the ribosome-activated GTPase reaction the Thr-61 anchor point is important for the correct transition state configuration.

In poly(Phe) synthesis EF-Tu[D80N]'s function appears as much impaired as EF-Tu[T61A]. Nevertheless, in partial reactions such as enzymatic binding (at 10 mM Mg^{2+}) and the (empty) ribosome-stimulated GTPase it behaved similar to wt, whereas at least the ribosome-stimulated GTPase of EF-Tu[T61A] was slowed considerably. Thus, it seems that these two mutants are inhibited in different steps of the elongation cycle. For EF-Tu[D80N] the rate-limiting step may be the (re)formation of the ternary complex, due to the

lower GTP affinity, whereas for EF-Tu[T61A] the slowest step may be the GTPase activation.

To resume, with the characterization of the mutants of Thr-25 published before and that of Thr-61 and Asp-80 presented in this work, we now have a fairly complete picture of the involvement of the network of magnesium binding residues in the GTPase. As a general trend we see that strong perturbation of the magnesium interaction reduces the intrinsic catalysis. This is in very good agreement with a transition state stabilization mechanism, in which the precise configuration of the various elements positioning the substrate and the nucleophile is essential. Whereas Thr-25 and Asp-80, both residues from "core" structural elements (helix α1 and strand β3, respectively), provide the main anchoring points for nucleotide binding, the peripheral effector region residue Thr-61 is weakly and transiently bound to the magnesium, playing however an important role in the transition state configuration. Inability of the residue at position 61 to bind Mg^{2+} decreases but does not preclude activation by the ribosome, while on the other hand suppressing the link from position 25 did not seem to decrease stimulation. Asp-80 appears to be involved in the tight regulation of the GTPase. The proposed role of the hydrophobic barrier composed of residues Val-20 and Ile-60 is not supported by our data. This makes the case for His-84 being a catalytic residue activating the nucleophilic water even weaker. As for the possibility that His-84 orients the water molecule in the transition state of the ribosome-induced GTPase, an induced drastic conformation change in the effector region would still be a prerequisite, and more data on the way the ribosome is involved are necessary to understand the mechanism of the physiological ribosome-stimulated GTPase of EF-Tu.

REFERENCES

1. Krab, I. M., and Parmeggiani, A. (1998) *Biochim. Biophys. Acta 1443*, 1−22.
2. Berchtold, H., Reshetnikova, L., Reiser, C. O., Schirmer, N. K., Sprinzl, M., and Hilgenfeld, R. (1993) *Nature 365*, 126−132.
3. Kjeldgaard, M., Nissen, P., Thirup, S., and Nyborg, J. (1993) *Structure 1*, 35−50.
4. Abel, K., Yoder, M. D., Hilgenfeld, R., and Jurnak, F. (1996) *Structure 4*, 1153−1159.
5. Polekhina, G., Thirup, S., Kjeldgaard, M., Nissen, P., Lippmann, C., and Nyborg, J. (1996) *Structure 4*, 1141−1151.
6. Swart, G. W. M., and Parmeggiani, A. (1989) *Biochemistry 28*, 327−332.
7. Rodnina, M. V., Pape, T., Fricke, R., Kuhn, L., and Wintermeyer, W. (1996) *J. Biol. Chem. 271*, 646−652.
8. Rodnina, M. V., Fricke, R., Kuhn, L., and Wintermeyer, W. (1995) *EMBO J. 14*, 2613−2619.
9. Voigt, J., Sander, G., Nagel, K., and Parmeggiani, A. (1974) *Biochem. Biophys. Res. Commun. 57*, 1279−1286.
10. Parmeggiani, A., and Sander, G. (1981) *Mol. Cell. Biochem. 35*, 129−158.
11. Ivell, R., Sander, G., and Parmeggiani, A. (1981) *Biochemistry 20*, 6852−6859.
12. Fasano, O., De Vendittis, E., and Parmeggiani, A. (1982) *J. Biol. Chem. 257*, 3145−3150.
13. Chinali, G., Wolf, H., and Parmeggiani, A. (1977) *Eur. J. Biochem. 75*, 55−65.
14. Scheffzek, K., Ahmadian, M. R., and Wittinghofer, A. (1998) *Trends Biochem. Sci. 23*, 257−262.
15. Cool, R. H., and Parmeggiani, A. (1991) *Biochemistry 30*, 362−366.
16. Scarano, G., Krab, I. M., Bocchini, V., and Parmeggiani, A. (1995) *FEBS Lett. 365*, 214−218.
17. Bourne, H. R., Sanders, D. A., and McCormick, F. (1991) *Nature 349*, 117−127.
18. Kjeldgaard, M., Nyborg, J., and Clark, B. F. C. (1996) *FASEB J. 10*, 1347−1368.
19. Harmark, K., Anborgh, P. H., Merola, M., Clark, B. F. C., and Parmeggiani, A. (1992) *Biochemistry 31*, 7367−7372.
20. Ahmadian, M. R., Kreutzer, R., Blechschmidt, B., and Sprinzl, M. (1995) *FEBS Lett. 377*, 253−257.
21. Jacquet, E., and Parmeggiani, A. (1988) *EMBO J. 7*, 2861−2867.
22. Krab, I. M., and Parmeggiani, A. (1999) *J. Biol. Chem. 274*, 11132−11138.
23. Deng, W. P., and Nickoloff, J. A. (1992) *Anal. Biochem. 200*, 81−88.
24. Anborgh, P. H. (1993) Elongation factor Tu from *E. coli*. Mechanism and action of a new specific antibiotic and site-directed mutagenesis, Ph.D. Thesis, Leiden University, Leiden, The Netherlands.
25. Inoue, H., Nojima, H., and Okayama, H. (1990) *Gene 96*, 23−28.
26. Swart, G. W. M., Parmeggiani, A., Kraal, B., and Bosch, L. (1987) *Biochemistry 26*, 2047−2054.
27. Parmeggiani, A., Singer, C., and Gottschalk, E. M. (1971) *Methods Enzymol. 20*, 291−302.
28. Anborgh, P. H., and Parmeggiani, A. (1991) *EMBO J. 10*, 779−784.
29. Anborgh, P. H., and Parmeggiani, A. (1993) *J. Biol. Chem. 268*, 24622−24628.
30. Louie, A., and Jurnak, F. (1985) *Biochemistry 24*, 6433−6439.
31. Abrahamson, J. K., Laue, T. M., Miller, D. L., and Johnson, A. E. (1985) *Biochemistry 24*, 692−700.
32. Jung, V., Wei, W., Ballester, R., Camonis, J., Mi, S., Van Aelst, L., Wigler, M., and Broek, D. (1994) *Mol. Cell. Biol. 14*, 3707−3718.
33. Miller, D. L., and Weissbach, H. (1977) in *Molecular mechanisms in protein biosynthesis* (Weissbach, H., and Pestka, S., Eds.) pp 323−373, Academic Press, New York.
34. Farrar, C. T., Halkides, C. J., and Singel, D. J. (1997) *Structure 5*, 1055−1066.
35. Halkides, C. J., Farrar, C. T., Larsen, R. G., Redfield, A. G., and Singel, D. J. (1994) *Biochemistry 33*, 4019−4035.
36. Zeidler, W., Egle, C., Ribeiro, S., Wagner, A., Katunin, V., Kreutzer, R., Rodnina, M., Wintermeyer, W., and Sprinzl, M. (1995) *Eur. J. Biochem. 229*, 596−604.
37. Scheffzek, K., Ahmadian, M. R., Kabsch, W., Wiesmüller, L., Lautwein, A., Schmitz, F., and Wittinghofer, A. (1997) *Science 277*, 333−338.
38. Rittinger, K., Walker, P. A., Eccleston, J. F., Smerdon, S. J., and Gamblin, S. J. (1997) *Nature 389*, 758−762.
39. Nassar, N., Hoffman, G. R. M., D, Clardy, J. C., and Cerione, R. A. (1998) *Nat. Struct. Biol. 5*, 1047−1052.
40. Al-Karadaghi, S., Ævarsson, A., Garber, M., Zheltonosova, J., and Liljas, A. (1996) *Structure 4*, 555−565.
41. Koradi, R., Billeter, M., and Wüthrich, K. (1996) *J. Mol. Graphics 14*, 51−55.

BI9909748