Elongation factor (EF) Tu Thr-25 is a key residue binding the essential magnesium complexed to nucleotide. We have characterized mutations at this position to the related Ser and to Ala, which abolishes the bond to Mg\(^{2+}\), and a double mutation, H22Y/T25S. Nucleotide interaction was moderately destabilized in EF-Tu(T25S) but strongly in EF-Tu(T25A) and EF-Tu(H22Y/T25S).

Binding Phe-tRNA\(^{\text{Phe}}\) to poly(U)ribosome needed a higher magnesium concentration for the latter two mutants but was comparable at 10 mM MgCl\(_2\). Whereas EF-Tu(T25S) synthesized poly(Phe), as effectively as wild type, the rate was reduced to 50% for EF-Tu(H22Y/T25S) and was, surprisingly, still 10% for EF-Tu(T25A). In contrast, protection of Phe-tRNA\(^{\text{Phe}}\) against spontaneous hydrolysis by the latter two mutants was very low. The intrinsic GTPase in EF-Tu(H22Y/T25S) and (T25A) was reduced, and the different responses to ribosomes and kirromycin suggest that stimulation by these two agents follows different mechanisms. Of the mutants, only EF-Tu(T25A) forms a more stable complex with EF-Ts than wild type. This implies that stabilization of the EF-Tu-EF-Ts complex is related to the inability to bind Mg\(^{2+}\), rather than to a decreased nucleotide affinity. These results are discussed in the light of the three-dimensional structure. They emphasize the importance of the Thr-25-Mg\(^{2+}\) bond, although its absence is compatible with protein synthesis and thus with an active overall conformation of EF-Tu.

Elongation factor (EF) Tu belongs to the superfamily of guanine nucleotide-binding proteins, factors involved in numerous cellular processes as carrier of information or biological components. Common characteristics in the family are the switching between active and inactive conformations depending on whether GTP or GDP is bound (1, 2), an intrinsic GTPase activity that can be stimulated several orders of magnitude by specific factors (GTPase-activating proteins), and the different responses to ribosomes and kirromycin indicating this fact.

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A double mutant EF-Tu (H22Y/T25S) isolated during the course of this study was also characterized and found to display intermediary properties, thus allowing to distinguish more precisely between specific effects of the two single substitutions.

**Materials and Methods**

**Biological Materials**—Ribosomes, EF-G, Phe-tRNA synthetase, and partially purified tRNA<sup>23</sup> (5 to 11%) were prepared from *E. coli* as reported (23, 24). Highly purified tRNA<sup>23</sup> was also obtained from Sigma.

**Construction, Overproduction, and Purification of EF-Tu Mutants**—To obtain rapid purification, EF-Tu wt and mutants were overproduced as fusion with glutathione S-transferase (25). Plasmid pGEX-2TgfA is a derivative of pGEX-2T (25) containing the *E. coli* tgfA gene coding sequence contiguous to the thrombin recognition sequence LVPRGS, where serine represents the first amino acid of EF-Tu (26). The very efficient cleavage by thrombin after arginine results in EF-Tu containing an additional N-terminal glycin, as compared with the native cellular EF-Tu, the N terminus of which is acetylated (27). All mutant proteins and also the control wt used in this study were produced in this way. Mutant T25A was generated by means of the polymerase chain reaction. Mutant T25S was obtained by the Unique Site Elimination method (28), using a kit from Amersham Pharmacia Biotech. Interestingly, when trying to construct the mutant T25S using either polymerase chain reaction or Unique Site Elimination methods with an 18 base mutagenic primer, we systematically obtained a double mutant H22Y/T25S resulting from the substitution of a flanking base. Use of a longer primer of 25 bases including codon 22 allowed us to obtain T25S alone.

The EF-Tu mutants were overproduced in *E. coli* DH5α grown in LB medium at 30 °C to 0.5–0.8 *A<sub>600</sub>* after which induction with 0.08 mM isopropyl-β-D-thiogalactopyranoside took place at 22 °C with incubations up to 40 h. Cells were disrupted by sonication in 50 mM Tris-HCl, pH 7.5, 110 mM KCl, 10 mM MgCl<sub>2</sub>, 7 mM ME and 10% glycerol (Buffer A). After centrifugation at 30,000 × *g* for 20 min, the supernatant was applied to a glutathione-Sepharose 4B column (Amersham Pharmacia Biotech; 2.5 ml of resin /g of cells), which was then extensively washed with Buffer A containing 0.1 M KCl, at which concentration EF-Ts cannot bind to the resin. EF-Tu was eluted with a linear KCl gradient to 400 mM. For the second method, the eluate from the glutathione affinity column was cleaved in situ and eluted at 4 °C with Buffer A supplemented with 30 μM GDP, 2.5 mM CaCl<sub>2</sub>, and 1 unit/ml thrombin at a low flow rate (approximately 0.3 ml/min) until no more protein was released. Thrombin in the eluent was inactivated by adding 0.1 mM phenylmethylsulfonyl fluoride. The obtained EF-Tu was further purified to remove residual EF-Ts, using either Q Sepharose chromatography or Superdex 75 gel filtration. For these mutants should not differ much from that of EF-Tu wt as far as possible. Glutathione S-transferase-EF-Tu was cleaved in situ and eluted at 4 °C with Buffer A containing 30 μM GDP, 2.5 mM CaCl<sub>2</sub>, and 1 unit/ml thrombin at a low flow rate (approximately 0.3 ml/min) until no more protein was released. Thrombin in the eluent was inactivated by adding 0.1 mM phenylmethylsulfonyl fluoride. The obtained EF-Tu was further purified to remove residual EF-Ts, using either Q Sepharose chromatography or Superdex 75 gel filtration. For the first method, the eluate from the glutathione affinity column was brought to a final GDP concentration of 250 μM and applied to a Q Sepharose FF column (6 ml). This was washed with Buffer A containing 250 μM GDP to induce the dissociation of EF-Tu-GDP, and 120 mM KCl at which concentration EF-Ts cannot bind to the resin. EF-Tu was eluted with a linear KCl gradient to 400 mM. For the second method, the affinity column eluate was concentrated by ultrafiltration (Amicon CentriFlo 25) to ~2 ml before loading on an Amersham Pharmacia Biotech Superdex 75 column, which was eluted with Buffer A containing 40 μM GDP. Pooled peak fractions from Q Sepharose or Superdex columns were concentrated by ultrafiltration, dialyzed against 50 mM Tris-HCl, pH 7.5, 60 mM NH<sub>4</sub>Cl, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 55% glycerol, 2 mM GDP, and stored at ~30 °C. Protein concentrations were 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 (29), and the GTPase activity was determined from liberation of [γ-<sup>32</sup>P]P, using the molybdate method (30). Nucleotide dissociation rates and affinities (15, 31–33), and protection of aa-tRNA by EF-Tu against spontaneous hydrolysis (34) were measured as described. Details are reported in the legends to the figures.

**Results**

**Production and Purification of EF-Tu Mutants**—Glutathione S-transferase-fused EF-Tu was expressed in *E. coli* DH5α cells grown in LB medium at 30 °C to 0.5–0.8 *A<sub>600</sub>*. At this cell concentration and temperature, induction by 0.08 mM isopropyl-β-D-thiogalactopyranoside for 6–8 h resulted in high level overproduction. However, whereas overproduced glutathione S-transferase-EF-Tu wt and T25S displayed good solubility (70–80%), yielding up to 2 mg of thrombin-cleaved EF-Tu/g cells (wt weight), mutants T25A and H22Y/T25S were about 10% soluble. Longer induction times (up to 40 h) at lower temperature during the induction phase (22 °C) somewhat increased the solubility. On the glutathione affinity column, some EF-Ts is always retained as a complex with EF-Tu, making a second purification step necessary, either by Q Sepharose chromatography or Superdex 75 gel filtration (see under “Materials and Methods”). This was particularly the case for the Ala mutant, which complexes very tightly with EF-Ts (75% EF-Tu-EF-Ts complex was still present after the first affinity step).

The presence of an additional glycine at the N-terminal extremity in EF-Tu wt did not modify its function in any of the activities tested in this work, including poly(Phe) synthesis.

**Interaction with GDP and GTP**—As shown in Table I, the single substitution T25S decreases the affinity for GDP already by a factor three, due to an increase in dissociation rate, whereas the association rate is comparable to wt. The T25A substitution and the addition of substitution H22Y to T25S have more dramatic effects: dissociation rates are 2 orders of magnitude higher than wt, and association rates decreased. Thus the affinity constants have μ instead of n M values, a difference of 3 orders of magnitude. The fast GDP dissociation causes a technical problem concerning the evaluation of the GDP binding capacity for mutants H22Y/T25S and T25A. The half-lives of 1 min and 25 s, respectively, are of the order of the filter washing time. Consequently the loss of nucleotide during filter washing is considerable; compared with wt around 50–60% of GDP is retained on filter by the double mutant EF-Tu(T25A) shows more variation, but still retains 25–50%.

Taking this and the order of the dissociation reaction into account, we estimated that the actual GDP binding capacity of these mutants should not differ much from that of EF-Tu wt (T25S proved identical). Consequently, we did not apply a correction factor in evaluating experiments in the other sections. For the properties presented in this section, a standardized washing procedure reduced experimental variation. Because the calculation of the constants in this section is based on relative experimental values at different time points or nucleotide concentrations, this technical difficulty does not affect the values in Table I.

A related reduction of filter retention compared with the theoretical value is already observed for EF-Tu wt-GTP filter binding, and is worse for the mutants. For EF-Tu(T25A), GTP retention by the filter-bound protein was even immeasurable. For the T25S single and double substitutions, GTP dissociation rates turned out to be 3–4-fold higher than for wt. Interestingly, although for the double mutant the affinity is nearly 6-fold lower and the association rate is not much changed.

| Table I  | Nucleotide interaction parameters |
|----------|----------------------------------|
|          | GDP                              | GTP                              |
|          | $k_{on}$                          | $K_d$                            |
|          | $s^{-1}$                          | $s^{-1}$                         | μM       |
|          | $10^4 k_{off}$                    | $10^4 K_d$                        | μM s<sup>-1</sup> |
| wt       | 3.9                               | 56.0                             | 0.0070   |
| T25A     | 270                               | 1.3                              | 2.1      |
| H22Y/T25S| 108                               | 1.1                              | 1.0      |
| T25S     | 10                              | 48.0                             | 0.0021   |
|          |                                   |                                  | 81       |
|          |                                   |                                  | 46.6     |
|          |                                   |                                  | 1.78     |
|          |                                   |                                  | 350      |
|          |                                   |                                  | 0.34     |
|          |                                   |                                  | 10.4     |
|          |                                   |                                  | 253      |
|          |                                   |                                  | 3.4      |
|          |                                   |                                  | 0.75     |

* Calculated as $k_{on} = k_{off}/K_d$, assuming a two-state association/dissociation reaction without intermediate steps.

A. Parmeggiani, unpublished observation.
compared with wt, for the T25S single substitution the affinity is actually slightly higher than for EF-Tu wt, and therefore the association rate is about 7-fold higher.

Activity in Poly(Phe) Synthesis—The most complete indication about the health of an EF-Tu mutant is its ability to support protein biosynthesis. We tested this activity by poly(U)-directed poly(Phe) synthesis at a magnesium ion concentration of 10 mM to ensure that the differences seen were not the result of insufficient saturation of EF-Tu with aa-tRNA, or insufficient binding to the ribosomal A site (see next paragraph); the GTP concentration was 400 μM, far above the affinity values measured at 0 °C, to convert the EF-Tu to the GTP form. As shown in Fig. 1, EF-Tu(T25S) could synthesize poly(Phe) as efficiently as wt. The double mutant EF-Tu(T25A/T25S) had a lower activity, about half that of EF-Tu wt. Surprisingly, EF-Tu(T25A), also, showed an activity that was significantly above the background level and was ~10% that of the EF-Tu wt elongation rate.

Enzymatic Binding of aa-tRNA to Programmed Ribosomes—The enzymatic binding of Phe-tRNA\textsuperscript{phe} to poly(U)-programmed ribosomes shows a modified dependence on Mg\textsuperscript{2+} concentration only for EF-Tu(T25S) and the double mutant, that of EF-Tu(T25S) being the same as that of EF-Tu wt (Fig. 2). EF-Tu(T25A) needed considerably (5–6 mM) more magnesium ion than EF-Tu wt to obtain 50% of the maximum binding of Phe-tRNA\textsuperscript{phe}; moreover, it was unable to reach the same plateau of binding as EF-Tu wt at any of the Mg\textsuperscript{2+} concentrations used, its maximum level corresponding to about 75% the control. In contrast, the double mutant displayed the same plateau level as EF-Tu wt, even though it needed approximately 3 mM more magnesium. The optimum enzymatic activity, defined as the highest ratio of enzymatic versus nonenzymatic binding occurred between 8 and 11 mM Mg\textsuperscript{2+}. This prompted our choice of a standard 10 mM magnesium ion concentration for the other assays.

Ester-bond Protection of aa-tRNA against Spontaneous Hydrolysis—The ability to participate in poly(Phe) synthesis and enzymatic binding, described in the previous section, established that the mutants have conserved either full (EF-Tu(T25S)) or at least partial activity (EF-Tu(H22Y/T25S) and EF-Tu(T25A)). On the other hand, the ability of EF-Tu to protect the ester-bound of aa-tRNA from spontaneous hydrolysis was strongly affected with the latter two mutants, as shown in Fig. 3, at 10 mM Mg\textsuperscript{2+}. In fact EF-Tu(T25S) induced the same protection as EF-Tu wt, whereas with the other two mutants, the half-life of Phe-tRNA\textsuperscript{phe} decreased from several hours to approximately 20 min, i.e. to virtually the same level as obtained with Phe-tRNA\textsuperscript{phe} in the absence of EF-Tu. This was surprising, especially in the case of the double mutant that was shown above to sustain a high rate of poly(Phe) synthesis and, at 10 mM Mg\textsuperscript{2+}, nearly the same enzymatic binding of aa-tRNA to the ribosome as EF-Tu wt.

Intrinsic GTPase Activity. Effect of aa-tRNA, Programmed Ribosomes and Kiorromycin—For measuring the intrinsic GTPase activity of these mutants, high GTP concentrations were used to saturate the EF-Tu with nucleotide, but for EF-Tu(T25A), this may not have been sufficient to ensure complete saturation. The intrinsic GTPase activity of the mutants versus that of EF-Tu wt was variably affected (Fig. 4A); for EF-Tu(T25S), it was comparable to that of EF-Tu wt and for the double mutant it was markedly reduced (~25%), whereas the rate of EF-Tu(T25A) was so low that it was difficult to determine. Interestingly, the presence of Phe-tRNA\textsuperscript{phe} which at magnesium concentrations ≥10 mM is known to hinder the hydrolysis of the EF-Tu wt-bound GTP (23, 35–37), decreased the activity of EF-Tu(T25S) by 60%, as for EF-Tu wt, whereas EF-Tu(H22Y/T25S) did not always show a clear reduction, and...
FIG. 3. Protection against spontaneous hydrolysis of the ester bond of Phe-tRNA<sub>Phe</sub> by EF-Tu. The concentration of [<sup>14</sup>C]Phe-tRNA<sub>Phe</sub> in solution was followed in time in the presence of EF-Tu wt (□), EF-Tu(T25A) (A), EF-Tu(H22Y/T25S) (C), or of EF-Tu(T25S) (•) or in absence of EF-Tu (×). The inset shows longer incubation times for EF-Tu wt and EF-Tu(T25S). Final reaction mixture (62 μl) contained 25 mM Hepes-KOH, pH 7.5, 60 mM NH₄Cl, 10 mM MgCl₂, 7 mM ME, 0.2 mM GTP, 1 mM PEP, 30 μg/ml pyruvate kinase, 1.7 μM [<sup>14</sup>C]Phe-tRNA<sub>Phe</sub>, specific activity, 405 dpm/pmole, and 3 μM EF-Tu as indicated. The reaction (at 30 °C) was started by adding 15 μl of buffer containing the [<sup>14</sup>C]Phe-tRNA<sub>Phe</sub> to 47 μl of mix with the other components, which had been preincubated at 30 °C for 15 min to convert EF-Tu into GDP. At the indicated times, 8-μl samples were taken and spotted onto glass fiber filters, and cold trichloroacetic acid-insoluble material on the filters was measured.

for EF-Tu(T25A) it gave low but measurable rates with a good correlation coefficient, at −5% of the wt intrinsic rate.

Kirimycin was able to enhance the GTPase activity of EF-Tu(T25S) and EF-Tu(H22Y/T25S), but not of EF-Tu(T25A), on which it was virtually inactive (Fig. 4B). Nevertheless, in poly(Phe) synthesis, the 50% inhibitory concentration for this mutant is even slightly lower (approximately 2 times) than for wt (not shown). Interestingly, kirimycin could compensate entirely the negative influence of the double mutation on the GTPase.

The presence of the ribosome, whether programmed with poly(U) or not (the latter not shown), markedly enhanced the GTPase activity of EF-Tu. In the absence of EF-Ts, the turnover rate of GTP hydrolysis of EF-Tu(T25S) is twice that of EF-Tu wt, as shown in Fig. 4C. This reflects the higher intrinsic GDP/GTP exchange rate of this mutant; in the presence of 50 nM EF-Ts, where the hydrolysis rate becomes limiting again, this difference disappears. The intrinsic GTPase dissociation rate of the double mutant and that of EF-Tu(T25A) are sufficiently high that the turnover GTPase rate is not limited by GDP/GTP exchange (not shown). The double mutant shows the same rate as wt in the absence of EF-Ts, but if compared with wt under conditions in which the catalysis is rate-limiting (in the presence of EF-Ts), it is lower by a factor of 5. Interestingly, EF-Tu(T25A) displays a clear GTPase in the presence of ribosomes, approximately 3% of the ribosome-stimulated wt rate (when the background rate is subtracted). The turnover rates of wt and the other mutants in the presence of ribosomes are 25–35 times their intrinsic GTPase rates. That of EF-Tu(T25S) is thus stimulated by ribosomes as much as the mutant is stabilized by EF-Ts.

**Dominant Negative-like Properties of the Two Mutants**

In Ras proteins, mutants of Ser-17, the residue equivalent to EF-Tu Thr-25, are known to cause a dominant negative phenotype, meaning that the mutant competes effectively with wt Ras for binding to its specific exchange factor, sequestering it and preventing activation of Ras. This was found to be associated with a strong decrease in the affinity of the mutants for GDP, and even more for GTP. To examine whether the same also holds for EF-Tu Thr-25 mutants, the stimulation by EF-Ts of the dissociation of radiolabeled GDP from EF-Tu wt was followed in the presence of increasing concentrations of nonlabeled EF-Tu-GDP. As shown in Fig. 5, the inhibition of the stimulation of EF-Ts on the EF-Tu wt [<sup>32</sup>P]GDP dissociation as a function of the concentration of added nonlabeled EF-Tu revealed a competitive inhibitory effect—i.e., an effect much stronger when than the “competitor” is EF-Tu wt—only in the case of EF-Tu(T25A). This is very likely due to a sequestration of EF-Ts resulting from the formation of a stable complex with EF-Tu(T25A), as was observed also during the purification of this mutant after overexpression in E. coli. Interestingly, no dominant inhibitory effect could be observed with EF-Tu(T25S) and EF-Tu(H22Y/T25S), even though for the double mutant the nucleotide affinity is affected to almost the same extent as for EF-Tu(T25A).

**DISCUSSION**

This work analyzes the effect induced on EF-Tu functions by substituting threonine 25, a strictly conserved α-helix 1 residue that flanks at the C-terminal side the P-loop (L1), and represents an essential element in the network coordinating the nucleotide-bound magnesium ion. Previous work of Hwang et al. (38) reported that substitution of this residue by isoleucine abolishes the interaction with the nucleotide. We decided to extend the analysis of the function of this crucial residue by introducing two other substitutions in the hope of correlating functional data with the recent impressive development of our knowledge of the three-dimensional structure of EF-Tu and its complexes. We also intended to compare the effects of this mutation with those reported for other guanine nucleotide-binding proteins on substituting the homologous residue.

The obtained results confirm the important structural role of this residue in the magnesium-substrate coordination, yet they show subtle effects not readily predictable from the current structural models. As for the nucleotide binding, the decrease in affinity of EF-Tu(T25A) for GDP by 3 orders of magnitude emphasizes the central role that the magnesium ion plays in the binding of the nucleotide to EF-Tu. EF-Tu(T25S) shows a 3-fold increase in dissociation rate, causing a subtle modification of the GDP affinity. This could be explained in terms of modification of the effector loop structure. In fact, two effector loop residues, Phe-46 and Ile-49 (Tyr-47 and Ile-50, respectively, in Thermus thermophilus) at both edges of the one-turn helix α'1 make hydrophobic contacts with the Thr-25 side chain methyl group, forming a kind of “hydrophobic clamp” (conserved motif (V/F)XX(L/V)) around it (Fig. 6, A and B). It represents the major interaction of this part of the effector region, in between the hypervariable first part and the conserved second part. Loss of this stabilizing hydrophobic interaction could unfavorably alter the structure of the effector loop, possibly affecting the water-mediated bond of Asp-50 with the Mg<sup>2+</sup>. In eukaryal and archael EF1αs, the conserved Thr at position 25 is replaced by a conserved Ser that lacks the β-carbon methyl group. Notably, in these factors the hypervariable part of the effector loop has in place of the hydrophobic clamp motif of prokaryal EF-Tu, a conserved hydrophobic motif containing several bulky aromatic residues. This might indicate a steric compensation for the absence of the methyl group at position 25.

The surprising effect of the supplementary mutation H22Y on the nucleotide affinity of EF-Tu(T25S) is more difficult to explain. Residue His-22 is part of the P-loop pointing toward L6.

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5 I. M. Krah, unpublished observation.
FIG. 4. GTPase activity: intrinsic and in the presence of Phe-tRNA$^{\text{Phe}}$ (A), kirromycin (B), and ribosomes (C). Liberation of $^{32}$Pphosphate from $[^{32}$P]GTP by EF-Tu wt (.), EF-Tu(T25A) (□ and ▲), EF-Tu(H22Y/T25S) (◇ and ◆), and EF-Tu(T25S) (○ and ●) in the various conditions. A, in absence (open symbols) or presence (filled symbols) of 3 μM $[^{14}$C]Phe-tRNA$^{\text{Phe}}$ (specific activity, 150 dpm/pmol). B, in absence (open symbols) or presence (filled symbols) of 50 μM kirromycin. C, in presence of 4 μM ribosomes, with only EF-Tu (open symbols) or with additionally 50 nM EF-Ts (filled symbols), or in absence of EF-Tu (×). Final reaction conditions were 50 mM Hepes-KOH, pH 7.5, 100 mM NH$_4$Cl, 10 mM MgCl$_2$, 7 mM Mg$_2$EDTA, 1 mM ATP, 50 μM $[^{32}$P]GTP (specific activity, 740–1500 cpm/pmol), 1 mM pep, 10 μg/ml pyruvate kinase, 2 μg/ml of the respective EF-Tu. EF-Tu-GTP was preformed by incubating this mix without the $[^{32}$P]GTP for 10 min at 30 °C. Reactions were started after further incubation with $[^{32}$P]GTP on ice for 10 min, by adding the stimulatory component and shifting the reaction to 30 °C. Appropriate-sized samples were taken at indicated times and quenched in 1 M HClO$_4$/3 mM KH$_2$PO$_4$, and free $^{32}$Pphosphate was determined by the molybdate/isopropyl acetate method (23).

FIG. 5. Inhibition of EF-Ts stimulation of EF-Tu(wt)$[^{3}$H]GDP dissociation by external EF-Ts-GDP. This figure shows how the stimulation of EF-Tu(wt)$[^{3}$H]GDP dissociation by EF-Ts is inhibited by the presence of increasing concentrations of competitor EF-Tu in the (unlabeled) GDP form: EF-Tu wt (□), EF-Tu(T25A) (▲), EF-Tu(H22Y/T25S) (○), or EF-Tu(T25S) (◆). The inhibition is defined as the decrease of the ratio of the observed $[^{3}$H]GDP dissociation rate ($k_{\text{obs}}$) and the EF-Ts-stimulated rate in the absence of competitor ($k_{\text{max}}$), both corrected for the intrinsic $[^{3}$H]GDP dissociation rate $k_{i}$ (i.e. in absence of EF-Tu). Values plotted are thus ($1 - (k_{\text{obs}} - k_{i})/k_{\text{max}} - k_{i}$) × 100%. Reaction conditions were 50 mM Hepes-KOH, pH 7.5, 60 mM NH$_4$Cl, 10 mM MgCl$_2$, 1 mM dithiothreitol, 30 mM EF-Tu(wt)$[^{3}$H]GDP (specific activity, 5800 dpm/pmol), 9 mM EF-Tu, 20 μM GDP, and indicated concentrations of various competitor EF-Ts-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 Fig. 2.

preceding helix α3. Substitution by the approximately 1.5 Ǻ longer tyrosine in the same orientation would cause a steric collision with the β-carbon of Met-112. Its accommodation would thus imply a displacement of L6, and consequently perhaps also of helix α3 (Fig. 6C). Noteworthy, in the EF-Tu-EF-Ts complex crystal structure, this helix has been displaced in the same direction by approximately 1.5 Ǻ as a consequence of the intrusion of EF-Ts Phe-81 in between EF-Tu helices α2 and α3. Thus, such a displacement might cause modifications similar to the sequence of interactions (from His-118 via Gln-114 to the P-loop) that Wang et al. (12) propose to lead to release of the nucleotide. Another possible effect of substitution H22Y could be the deformation of the P-loop, hindering nucleotide accommodation. The slightly higher magnesium requirement for enzymatic binding indicates that some perturbation indeed occurs at the level of the magnesium binding elements in the double mutant and not with the single substitution T25S.

A related observation deserves some attention. In Ras proteins, mutations of the position equivalent to EF-Tu Thr-25 are known to be associated with dominant negative phenotypes (19–22). This has been shown in vitro for Ras2p(S24N) to be associated with increased stability of the complex of Ras2p with its exchange factor (22, 39), thus leading to sequestration of the guanine nucleotide exchange factor. Interestingly, here we describe two mutants of EF-Tu (EF-Tu(H22Y/T25S) and EF-Tu(T25S)) that have a comparable decrease in affinity for GDP, whereas only one, EF-Tu(T25A), shows dominant negative-like behavior through increased stability of the complex with EF-Ts. Nevertheless the stimulation by EF-Ts of GDP dissociation on EF-Tu(H22Y/T25S) is not less efficient than with wt (not shown). This indicates that the interaction of the magnesium ion with Thr-25 plays a central role in the mechanism of dissociation of the EF-Tu-EF-Ts complex. In the crystal structure of the complex, the phosphate cavity is not completely open due to a peptide flip in the P-loop. If the stable interaction Mg$^{2+}$-Thr-25(OH) cannot be formed, the insertion of the nucleotide into the binding site and the release of EF-Ts has apparently a much lower chance of succeeding. This observation may also have implications for the reverse process, the dissociation of EF-Tu-nucleotide by EF-Ts, in which breaking of the Mg$^{2+}$-Thr-25(OH) bond could represent the major energetic barrier. This would emphasize the importance of the displacement of magnesium in the mechanism of stimulation of EF-Tu-GDP dissociation by EF-Ts, in agreement with the model of Kawashima et al. (11).

Concerning the influence of the various mutations on the GTPase activity, the most striking observation is the different effects on the stimulation of the EF-Tu GTPase by kirromycin and ribosomes. The negative influence of the double mutation on the intrinsic GTPase can be completely compensated by kirromycin, whereas the GTPase of EF-Tu(T25A) is completely insensitive to the antibiotic, although it still interacts. In contrast, ribosomes are shown here to stimulate all mutants and
This is probably related to a different mechanism of stimulation of the catalysis by the two agents, which are known to have an additive effect (15). The ribosome stimulation may be somehow related to that proposed for the GTPase-activating protein stimulation of small GTPases, even though some caution in comparing the two systems is in order (6). Our findings suggest that the enhancing effect of the ribosome is not critically dependent on the tight interaction of the magnesium ion with the core of EF-Tu, although the magnesium ion is essential for achieving the correct binding of nucleotide and the intrinsic GTPase. This is in evident contrast with the effect of the antibiotic kirromycin that we show here to stimulate the GTPase only if the Mg$^{2+}$-25(OH) bond is intact, thus indicating a different mechanism of action from that of the ribosome. The location and mode of binding of the antibiotic to EF-Tu are not yet precisely defined. Whereas resistance mutations were mapped to the interface of domains 1 and 3 (40, 41), the known competition between EF-Ts and kirromycin for binding to EF-Tu could rather implicate elements from the EF-Tu-EF-Ts contact area (L1(P-loop), L6-$\alpha_3$, and $\alpha_4$ (11)) in kirromycin binding (6). Consequently, kirromycin could have a direct or indirect interaction with the GTPase center, perhaps acting through the P-loop. There it might strengthen the hydrogen bond of the Asp-21 main chain NH toward the $\beta$- bridging oxygen, which was suggested for p21 to play a major role in the intrinsic GTPase (42).

Concerning the interaction with tRNA, we have observed various effects. First, the activities in poly(Phe) and enzymatic binding indicate that EF-Tu(T25S) has conserved full ability to bind aa-tRNA, that also the double mutant at 10 mM Mg$^{2+}$ is like wt, and that even EF-Tu(T25A) has conserved at least partial activity. In surprising contrast is the complete lack of protection against spontaneous hydrolysis of the aa-tRNA ester bond by the latter two mutants. We interpret this to indicate that whereas the overall binding of aa-tRNA may not be dramatically affected, the interaction with the aminoacyl end of the tRNA is anomalous for these mutants. Interestingly, this recalls the situation observed for wt EF-Tu in the presence of the antibiotic enacyloxin IIa (43). A combination of factors probably contributes to the impaired rate of poly(Phe) synthesis with two of the three mutants: the anomalous ternary complex, the reduced efficiency of the GTPase center, and in the case of EF-Tu(T25A) also the more stable complex with EF-Ts.

To conclude, our results underline the importance of the magnesium binding by Thr-25 in the functioning of EF-Tu, whereas on the other hand the ability of EF-Tu to participate in protein synthesis proves conserved to some extent even in the absence of this bond. This shows that modification of the magnesium-nucleotide binding network, although drastic, as in the case of substitution of the key component Thr-25 by Ala, is still compatible with an active overall conformation of the EF-Tu Waals surface, surrounding the Thr-25 side chain methyl group, indicated similarly in yellow. Magnesium (dark green) and the nucleotide are also visualized. C, close-up of the contacts between the effector region of EF-Tu, Thr-25, the nucleotide, and the magnesium ion. A, E. coli EF-Tu in the GDP-form (9); B, T. thermophilus EF-Tu in the GTP form (7). The effector region is highlighted in pale blue, with residues forming the hydrophobic clamp shown as wireframe plus transparent blue Van der Waals surface, and Met-112 of EF-Tu GDP shown in yellow. The two loops L1 (P-loop) and L6 are represented as a backbone wireframe, with the side chains of His-22 and Met-112 of EF-Tu GDP shown as well. Superimposed on His-22 is a tyrosine (purple) in the same orientation. Its terminal OH group is surrounded by a transparent Van der Waals surface, as are atoms from Ala-110 and Met-112 in loop L6 in which it would clash. Representations were made using Molscript and Raster3D (44, 45).
molecule. It also emphasizes that although the Mg\(^{2+}\)-Thr-25(OH) bond contributes greatly to the stability of nucleotide binding, other interactions are important too.

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