Mutants of EF-Tu defective in binding aminoacyl-tRNA

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Abstract **Five single amino acid substitution variants of EF-Tu from *Salmonella typhimurium* were tested for their ability to promote poly(U)-translation in vitro. The substitutions are Leu^{120}Gln, Gln^{124}Arg and Tyr^{160} (Asp or Asn or Cys). They were selected by their kirromycin resistant phenotypes and all substitutions are in domain I at the interface between domains I and III of the EF-Tu·GTP configuration. The different EF-Tu variants exhibit a spectrum of phenotypes. First, k_{cat}/K_M for the interaction between ternary complex and the programmed ribosome is apparently reduced by the substitutions Leu^{120}Gln, Gln^{124}Arg and Tyr^{160}Cys. Second, this reduction is caused by a defect in the interaction between these EF-Tu variants and aminoacyl-tRNA during translation. Third, in four cases out of five the affinity of the complex between EF-Tu·GTP and aminoacyl-tRNA is significantly decreased. The most drastic reduction is observed for the Gln^{124}Arg change, where the association constant is 30-fold lower than in the wild-type case. Fourth, missense errors are increased as well as decreased by the different amino acid substitutions. Finally, the dissociation rate constant (k_d) for the release of GDP from EF-Tu is increased 6-fold by the Tyr^{160}Cys substitution, but remains unchanged in the four other cases. These results show that the formation of ternary complex is sensitive to many different alterations in the domain I-III interface of EF-Tu.**

Key words: Translation in vitro; Kirromycin; EF-Tu; Ternary complex

1. Introduction

Elongation factor Tu (EF-Tu) is a protein of 393 amino acids folded into three distinct structural domains [1,2] and forms a ternary complex with GTP and aa-tRNA. The ternary complex participates in protein biosynthesis by mediating the interaction of aa-tRNA with the ribosomal A site, accelerating very significantly the rate of aa-tRNA binding to the ribosome, and reducing missense errors by more than 2 orders of magnitude. These activities of EF-Tu depend on the nature of the bound guanine nucleotide [3]. In its GTP conformation EF-Tu has a high affinity for aa-tRNA with an association constant in the range of 10^7 M^{-1} [4,5]. The (cognate) interaction between an mRNA codon in the A-site and the anticodon of aa-tRNA in ternary complex with EF-Tu·GTP is rapidly followed by hydrolysis of GTP on EF-Tu [6]. GTP hydrolysis changes the conformation of EF-Tu and causes its subsequent, rapid release from the ribosome. The affinity of EF-Tu for aa-tRNA is 2 orders of magnitude lower when the factor is in its GDP-rather than its GTP-configuration [7]. A second elongation factor, EF-Ts, catalyses the exchange of GDP to GTP on EF-Tu, thus reactivating EF-Tu so that it can again bind aa-tRNA and form the translationally active ternary complex [8].

Mutation studies and physico-chemical analyses suggest that residues in all three domains of EF-Tu influence its binding of aa-tRNA. Site-directed mutagenesis of conserved His residues in domain I (His^{66} and His^{118}) show that alterations at these positions reduce the affinity of EF-Tu for aa-tRNA [9,10]. The residues Lys^{208} and Lys^{237} in domain II of EF-Tu have been crosslinked, in the presence of kirromycin, to 3′ oxidised tRNA [11]. Residues in EF-1α equivalent to residues (260–263) in domain II of *E. coli* EF-Tu are crosslinked to, and protected from protease digestion by, aa-tRNA [12]. The kirromycin resistant *tufAa* allele in *E. coli*, Glu^{378}Lys in domain III of EF-Tu, is defective in binding aa-tRNA [5,13]. Truncated EF-Tu, lacking either domain I, or both domains II and III, shows no interaction with aa-tRNA [14,15]. The crystal structure of the ternary complex of EF-Tu·GTPc·aa-tRNA has recently been solved [16] opening the possibility to evaluate at a structural level the effects of mutations in EF-Tu on interactions with aa-tRNA.

Kirromycin, an antibiotic which binds EF-Tu, inhibits the release of EF-Tu·GDP from the ribosome [17]. Selections for spontaneous kirromycin resistant mutations in *Salmonella typhimurium*, have identified 13 different substitutions clustering in the domain I-III interface [13]. We have studied the influence of five of these mutants in domain I on translation in vitro and find significant effects on aa-tRNA binding to EF-Tu·GTP as well as on missense error levels in poly(U)-translation.

2. Materials and methods

2.1. Chemicals

Poly(U), GTP, ATP and GDP were purchased from Pharmacia, Sweden. Phospho*enol*pyruvate (PEP), putrescine, spermidine, myokinase (MK) (EC 2.7.4.1), pyruvate kinase (PK) (EC.2.7.1.40), L-phenylalanine and L-leucine were products of Sigma, St Louis, USA. [^{3}H]-and [^{14}C]phenylalanine as well as [^{3}H]leucine and [^{3}H]guanosine 5′-diphosphate were from Amersham, Bucks, UK. All other chemicals of analytical grade were from Merck, Darmstadt, Germany.

2.2. Buffers

Polymix buffer [18] contains 5 mM magnesium acetate, 0.5 mM $CaCl_2$, 5 mM NH_4Cl, 95 mM KCl, 8 mM putrescine (pH 7.5), 1 mM spermidine, 5 mM potassium phosphate (pH 7.3) and 1 mM dithioerythritol (DTE). 10 times concentrated polymix buffer (10×pmix) was prepared and stored without potassium phosphate (to avoid precipitation) and DTE. The correct working strength of polymix was obtained by adding from stock solutions of 10×pmix, 100 mM potassium phosphate (20×KP) and 50 mM DTE in the experiments.

2.3. Strains and biochemicals

tRNA^{Phe}, Phe-tRNA^{Phe} synthetase (PRS) and Leu-tRNA^{Leu} synthetase (LRS) were purified from *E.coli* MRE-600 cells according

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to Ehrenberg et al. [19]. Ribosomes, wild-type EF-Tu and EF-G were prepared from either *E.coli* 017 [20] or from *S. typhimurium* LT2 [21], EF-Ts was prepared from 017 and the altered EF-Tu's from LT2 cells. Purification procedures and storage conditions are described by Ehrenberg et al. [19]. The EF-Tu preparations were modified according to Hughes [22].

2.4. EF-Tu concentration

The purity of all EF-Tu preparations was higher than 95%, as judged from SDS-PAGE. The protein content of EF-Tu was estimated according to Bradford [23]. As protein standard was used an EF-Tu with its concentration determined by amino acid analysis. Nucleotide exchange assays [4,24], that measure the concentration of EF-Tu active in GDP-binding, showed EF-Tu concentrations identical with those obtained by the Bradford method.

2.5. Poly(U)-translation assays

Poly(Phe)-synthesis rates were measured at 37°C according to Ehrenberg et al. [19].

2.5.1. Assay 1. k_{cat} and K_M values for the interaction between ternary complex and ribosomes were obtained from translation assays at different EF-Tu concentrations. A factor mix and a ribosome mix were separately prepared in polymix buffer. The factor mix contained 2 mM ATP, 20 mM PEP, 2 mM GTP, 0.6 mM [^{14}C]Phe, and (per 50 μl) 5 μg PK, 0.3 μg MK, 100 pmol EF-Ts, 200 pmol EF-G, 700 pmol tRNA^{Phe}, 200 units PRS (one unit of PRS charges one pmol of tRNA^{Phe} per s). 10 μl aliquots of EF-Tu dilutions (10–300 pmol) in polymix buffer were added to 50 μl aliquots of the factor mix. The ribosome mix contained (per 40 μl) 10 pmol of ribosomes active in elongation, 50 pmol NAc-[^{3}H]Phe-tRNA^{Phe} and 20 μg poly(U). Factor and ribosome mixes were pre-incubated at 37°C for 10 min. Translation was started by adding 40 μl of ribosome mix to each of the factor mixes. After incubation times in the range 5–25 s, chosen to make the poly(Phe) chains of similar length at different elongation rates [19], the reactions were stopped by addition of 5 ml 5% TCA. The samples were then analysed for extent of poly(Phe) synthesis (from [^{14}C]Phe) and for amount of elongating chains (from NAc-[^{3}H]Phe) [19,25].

2.5.2. Assay 2. Determination of translation rates at different concentrations of Phe-tRNA^{Phe}. Separate factor and ribosome mixes were prepared as in assay 1, except that the amount of EF-Tu in the factor mix now was fixed at 10 pmol, while that of Phe-tRNA^{Phe} was varied from 50 to 2000 pmol and the assay incubation times were varied in the range 10–30 s [4,5].

2.6. Binding constant for the complex between aa-tRNA and EF-Tu·GTP

The spontaneous deacylation rate constant for free aa-tRNA (k_f) is much faster than for aa-tRNA in complex with EF-Tu·GTP (k_b) [4,5,26,27]. The fraction (T_f/T_0) of free (T_f) divided by total (T_0) aa-tRNA was estimated from the average deacylation rate constant (k), obtained for different total concentrations of EF-Tu·GTP (Tu_0) at a fixed T_0 [4,27]:

$$k = k_f \cdot \frac{T_f}{T_0} + k_b \cdot \frac{T_b}{T_0} = (k_f - k_b) \cdot \frac{T_f}{T_0} + k_b$$

This relation is valid when the deacylation rate constants k_b and k_f are small in relation to the rate constant for aa-tRNA dissociation from EF-Tu·GTP [4,5,27]. $T_b/T_0 = (1 - T_f/T_0)$ is the ratio between aa-tRNA bound to EF-Tu·GTP (T_b) and T_0. Experimental determination of k_f, k_b and k values at each point in the EF-Tu titration (specified below) gives T_f/T_0 and T_b/T_0 as

$$\frac{T_f}{T_0} = \frac{k - k_b}{k_f - k_b}, \frac{T_b}{T_0} = \frac{k_f - k}{k_f - k_b}$$

2.6.1. Deacylation protection assay. A tRNA charging mix was prepared on ice in polymix buffer. It contained 0.3 mM [^{14}C]Phe, 1 mM ATP, 10 mM PEP, 1 mM GTP, and (per 80 μl) 5 μg PK, 0.3 μg MK, 4 units of PRS, and either 100 pmol of Phe-tRNA^{Phe} (for wild-type and the EF-Tu variant Tyr^{160}Asp), or 400 pmol of Phe-tRNA^{Phe} (for the EF-Tu variants Tyr^{160}Asn, Tyr^{160}Cys, Gln^{124}Arg and Leu^{120}Gln). For binding in the presence of kirromycin (dissolved in 0.5% methanol) the charging mix contained 1000 pmol (w.t. EF-Tu) or 10000 pmol (EF-Tu variants ^{120}Gln and ^{160}Cys) kirromycin. In control assays only methanol or only polymix buffer were added instead of kirromycin. Aliquots of EF-Tu (10 μl) containing 0–1400 pmol (w.t., Tyr^{160}Asp) or 0–3000 pmol (Tyr^{160}Asn, Tyr^{160}Cys, Gln^{124}Arg, Leu^{120}Gln) were added to the charging mixes (80 μl). Then, the mixes were preincubated for 5 min at 37°C for full charging of tRNA with [^{14}C]Phe. Incubation was started by adding 10 μl (100 pmol) of [^{3}H]Phe to each charge mix. The reactions were stopped after 20 min by adding 5 ml ice-cold 5% TCA, and the samples were analysed for the amounts of tRNA charged with [^{3}H]Phe and [^{14}C]Phe. The amount of [^{3}H]Phe-tRNA^{Phe} (a_h) increases with the incubation time T as

$$a_h = a_c \cdot (1 - e^{-kT})$$

The level, a_c, of [^{14}C]Phe-tRNA^{Phe} was constant throughout the incubation time T, in contrast to earlier methods [27], because deacylation was immediately followed by reacylation with [^{14}C]Phe and [^{3}H]Phe. The [^{3}H]Phe, added at the start of incubation, had high specific activity and much lower concentration than [^{14}C]Phe.

Measurement of a_c and a_h at the incubation time T gives k as

$$k = -\frac{1}{T} \cdot \ln\left(1 - \frac{a_h}{a_c}\right)$$

Calibration of the specific activities of [^{14}C]Phe and [^{3}H]Phe, to obtain a_h/a_c as pmol [^{3}H]Phe per pmol [^{14}C]Phe, was performed by aminoacylating tRNA^{Phe} with the [^{14}C]Phe and [^{3}H]Phe amino acids mixed already at the start of the aminoacylation reaction.

Accurate values of k were obtained also for small values of a_h/a_c, which significantly reduced the incubation time T in relation to previous techniques [27]. In addition, the constant [^{14}C]Phe charging level a_c led to simple and accurate data evaluation.

2.7. Missense error rates in vitro

Missense error rates were measured in EF-Tu·GTP titrations with bulk tRNA, where Leu-tRNA^{Leu} competes with Phe-tRNA^{Phe} in poly(U)-translation.

2.7.1. Factor mixes. Factor mixes were prepared on ice in balanced polymix buffer with 2 mM GTP, 20 mM PEP, 2 mM ATP, 0.6 mM [^{14}C]Phe, 0.08 mM [^{3}H]Leu and (per 50 μl) 5 μg MK, 0.3 μg PK, 100 pmol EF-Ts, 200 pmol EF-G, 100 pmol tRNA^{Phe}, 200 pmol tRNA^{Leu}, 100 units PRS, 10 units LRS. To 50 μl aliquots of these mixes were added 10 μl aliquots of EF-Tu (50–1200 pmol) in polymix buffer. The mixes were preincubated for 10 min at 37°C.

2.7.2. Ribosome mixes. One ribosome mix was prepared on ice as in section 2.5 with NAc-[^{14}C]Phe-tRNA^{Phe} replacing NAc-[^{3}H]Phe-tRNA^{Phe}. A second ribosome mix, where polymix buffer replaced poly(U), was prepared for missense error backgrounds. The ribosome mixes were preincubated for 10 min at 37°C.

2.7.3. Missense error assay. Translation was started by adding 40 μl aliquots from the ribosome mix to each factor mix (60 μl). After 30 s incubation at 37°C the reactions were stopped by 5 ml 5% TCA, and the samples analysed according to Ehrenberg et al. [19].

2.7.4. RNA charging. For charging determinations, factor mixes (50 μl) were prepared as above, but without EF-Tu and with either LRS (for Phe-tRNA^{Phe} levels) or PRS (for Leu-tRNA^{Leu} levels) replaced by polymix buffer, to eliminate spillover between the ^{14}C and ^{3}H channels of the counter. After 10 min incubation, charging levels were measured as in section 2.6 [19].

3. Results

3.1. Translation rate as a function of EF-Tu concentration

Titrations were made with wild-type and mutant species of EF-Tu at a constant ribosome concentration (10 pmol active in 100 μl). The maximum rate for the complete ribosome cycle (k_{cat}) is similar for all studied variants of EF-Tu, and was close to 10 amino acids per ribosome per second (Fig. 1). The k_{cat}/K_M value for ternary complex association with the ribosome is similar for the wild-type and the EF-Tu mutants ^{160}Asp and ^{160}Asn at close to 1.7×10^7 (M^{-1} s^{-1}) (Fig. 1A, Table 1). However, the other mutant EF-Tu's tested, ^{160}Cys, ^{124}Arg and ^{120}Gln, apparently have significantly reduced k_{cat}/K_M values (1.25, 0.8, and 0.8×10^7 M^{-1} s^{-1}, respectively) (Fig.

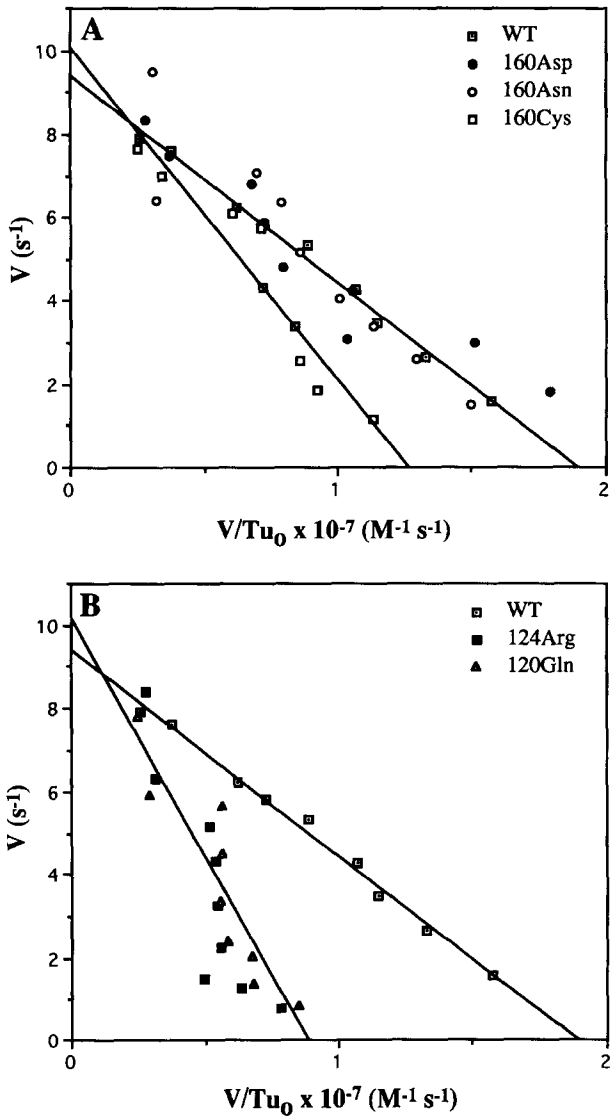

Fig. 1. Eadie-Hofstee plots for the rate of elongation (v) of poly(U) translating ribosomes for different total concentrations of EF-Tu (Tu_0). v is the extent of poly(Phe) synthesis (measured from hot TCA precipitable [^{14}C]Phe) normalized to the number of poly(Phe) chains (measured from hot TCA precipitable NAc-[^{3}H]Phe) and divided by the actual incubation time (section 2). The intercept at the y-axis gives k_{cat} and the intercept at the x-axis gives k_{cat}/K_M for poly(Phe) elongation. (A) Wild-type and the EF-Tu variants ^{160}Asp, ^{160}Asn and ^{160}Cys. (B) Wild-type and the variants ^{124}Arg and ^{120}Gln.

1A,B and Table 1). Further analyses were carried out to investigate if it is the association between ternary complex and the ribosome which is affected by the mutations, or whether they cause a defect in binding of aa-tRNA to EF-Tu·GTP.

3.2. Translation as a function of tRNA concentration

This assay was made to test whether the apparent reduction in k_{cat}/K_M values for some of the mutant ternary complex interactions with the ribosome could be overcome by increasing the concentration of aa-tRNA, which could enhance the fraction of EF-Tu in the ternary complex in spite of a perturbed binding. The assays were carried out for w.t. EF-Tu and for the variants ^{120}Gln, ^{124}Arg, ^{160}Cys, which showed apparent defects in k_{cat}/K_M (section 3.1). Translation was run at fixed, low concentrations of EF-Tu (10 pmol per 100 μl), and varied concentrations of Phe-tRNA^{Phe} (50–2000 pmol per 100 μl). In each case, titration to high values of aa-tRNA abolished the apparent defect in ternary complex interaction with the ribosome (Fig. 2). This shows that the major defect of these mutants is in the binding of aa-tRNA to form ternary complex.

3.3. Binding of EF-Tu·GTP to aa-tRNA

To test more directly defects in the binding of aa-tRNA by the mutant EF-Tu's we measured equilibrium binding constants (section 2). In the Scatchard [28] plots in Fig. 3, the equilibrium binding constant between EF-Tu·GTP and aa-tRNA is the intercept on the y-axis, while the apparent stoichiometry (n) of EF-Tu·GTPs bound to aa-tRNA is the inverse of the intercept on the x-axis. The association constant (K_A) for wild-type EF-Tu·GTP and Phe-tRNA^{Phe} is 0.75×10^7 M^{-1} (Fig. 3A) and the stoichiometry, n, is close to 2, in agreement with published values [4,5]. In contrast, K_A for wild-type EF-Tu·GDP is about 200-fold lower and is only 0.37 10^5 M^{-1}, confirming earlier results [7,27,29] and demonstrating the importance of the EF-Tu·GTP form for the interaction with aa-tRNA. Kirromycin increases the association constant K_A about 2-fold (K_A = 1.32 10^7 M^{-1}). At the same time, no effect of kirromycin on the K_A values for the mutants ^{120}Gln and ^{160}Cys was observed. In addition, we found that even very high concentrations of EF-Tu·GDP·kirromycin complex (2×10^{-5} M) did not protect aa-tRNA from deacylation.

The mutant ^{160}Asp has a K_A value of 0.71×10^7 M^{-1}, as in the wild-type case. The mutants ^{160}Asn and ^{160}Cys have association constants reduced to 0.4 and 0.25×10^7 M^{-1}, respectively (Fig. 3B, Table 1). The most severe defects were seen with the mutants ^{120}Gln and ^{124}Arg, where K_A is reduced to 0.12 and 0.025×10^7 M^{-1}, respectively (Fig. 3C, Table 1). The association constant for the mutant ^{124}Arg was too weak to give an accurate Scatchard value, and was therefore estimated with the stoichiometry n fixed and equal to 2, as found for the other EF-Tu variants (Fig. 3C).

3.4. Missense errors

Missense errors were calculated from the frequency of the incorporation of near cognate Leu-tRNA^{Leu} in response to poly(U)-programmed ribosomes and in competition with Phe-tRNA^{Phe} (section 2). In titrations with EF-Tu any differences in the ability of the competing aa-tRNA's (tRNA^{Leu} and tRNA^{Phe}) to form ternary complex will eventually be overcome at high EF-Tu concentrations. Thus, at low levels of EF-Tu variations in error may reflect competition by the

Table 1
(A) Binding of aa-tRNA to EF-Tu·GTP, and (B) k_{cat}/K_M of the ternary complex ribosome interaction

EF-Tu variant	(A) $K_A \times10^{-7}$ (M^{-1})	(B) $k_{cat}/K_M\times10^{-7}$ (M^{-1} s^{-1})
WT(GTP)	0.75	1.8
WT(GDP)	0.0037	–
WT(GTP·Kir)	1.32	–
^{160}Asp	0.71	1.8
^{160}Asn	0.4	1.7
^{160}Cys	0.25	1.25
^{124}Arg	0.025	0.8
^{120}Gln	0.12	0.8

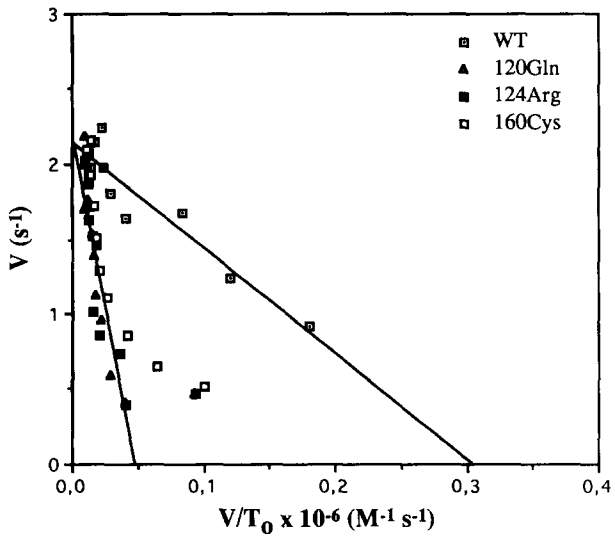

Fig. 2. Eadie-Hofstee plots for the rate of elongation (v) at limiting EF-Tu concentration (10^{-7} M) at different concentrations of Phe-tRNA^{Phe} for wild-type EF-Tu and for the variants ^{160}Cys, ^{124}Arg and ^{120}Gln. v is determined as in Fig. 1. The intercept at the y-axis shows the rate of poly(Phe) elongation at a rate-limiting EF-Tu concentration and at saturating concentrations of Phe-tRNA^{Phe}, where differences in tRNA binding to the different EF-Tu variants are eliminated. The common intercept in all four cases (only one is shown for clarity) shows that the deficiency in k_{cat}/K_M for these three EF-Tu variants (Fig. 1) is caused by inefficient ternary complex formation and not by impaired interaction by the corresponding ternary complexes and the ribosome. The intercept at the x-axis shows how the interaction between aminoacyl-tRNA and the different EF-Tu variants affects poly(Phe) synthesis at rate-limiting concentrations of Phe-tRNA^{Phe} and EF-Tu.

aminoacyl-tRNAs for EF-Tu to form active ternary complex. At high levels of EF-Tu, in contrast, the error level only reflects the relative efficiency by which cognate and non-cognate ternary complexes compete for the poly(U)-programmed ribosome. The error rate associated with the wild-type EF-Tu was estimated to be 5×10^{-4}, in agreement with published values [25,30]. For the mutants of EF-Tu the pattern is quite complex. For all concentrations of EF-Tu, ^{160}Cys and ^{160}Asp have reduced error levels (2 and 3×10^{-4}, respectively) (Fig. 4A), ^{124}Arg has a wild-type error level (5×10^{-4}) and ^{120}Gln has an increased error level (9×10^{-4}) (Fig. 4B). The constancy of these error levels at varying EF-Tu concentrations indicates that in these cases the affinities of Leu-tRNA^{Leu} and Phe-tRNA^{Phe} to EF-Tu are quite similar. In contrast, ^{160}Asn has error levels which increase with increasing EF-Tu concentrations, indicating that this EF-Tu variant has a significantly lower affinity for the Leu-tRNA^{Leu} than for Phe-tRNA^{Phe}. ^{160}Asn has an error level of more than 10^{-3} at saturating EF-Tu concentrations (Fig. 4A). This suggests that this mutation not only weakens the binding of Leu-tRNA^{Leu} to EF-Tu in relation to the binding of Phe-tRNA^{Phe} to the factor (the errors increase significantly with increasing EF-Tu concentration in contrast to the wild-type case where they are approximately constant), but also makes ternary complex with Leu-tRNA^{Leu} a more efficient competitor to Phe-tRNA^{Phe} containing ternary complex in poly(U)-translation.

One of the EF-Tu variants, ^{160}Cys, has a GDP exchange rate constant that is significantly higher than for wild-type EF-Tu (0.06 and 0.01 s^{-1}, respectively), showing that amino acid substitutions at the interface between domains I and III may, conditionally, affect the binding of GDP to EF-Tu. Each of the other four mutant EF-Tu's has a GDP exchange rate which is not significantly different from the wild-type.

4. Discussion

Substitution mutations at three amino acid sites in domain I of EF-Tu, Leu^{120}, Gln^{124}, Tyr^{160} give rise to a kirromycin resistant phenotype [13]. The side chains of these three amino acids are aligned and in contact, Gln with Leu and Leu with Tyr, and all point into the domain I-III interface in both the GTP and GDP conformations of EF-Tu [1,31]. In an EF-Tu-limited translation elongation assay under standard conditions [19] three of the mutants, ^{120}Gln, ^{124}Arg, and ^{160}Cys have an apparently reduced k_{cat}/K_M for the interaction of cognate ternary complex with the ribosome during translation (Fig. 1B, Table 1). Mutants in EF-Tu can exert such effects either by influencing the efficiency with which ternary complex is formed, or the rate at which ternary complex interacts with and delivers aa-tRNA to the ribosome. This apparent k_{cat}/K_M reduction could in all cases be reversed by enhancing the concentration of Phe-tRNA^{Phe}, showing that the defect causing it in each of these mutants (^{120}Gln,^{124}Arg and^{160}Cys) is in the formation of ternary complex, rather than in the cognate interaction between ternary complex and the ribosome.

We probed this defect more directly by measuring the equilibrium binding constant between each of the mutant EF-Tu's and Phe-tRNA^{Phe}. The substitution mutations ^{160}Asn and ^{160}Cys reduce somewhat the binding to Phe-tRNA^{Phe}, but the most dramatic effects are caused by ^{120}Gln and ^{124}Arg: their association constants are reduced to one eighth and one thirtieth, respectively, of the wild-type level (Fig. 3). The magnitude of these binding effects is consistent with the relative magnitude of the effects seen with these mutants in the translation assays (Fig. 2).

It is known from previous work that substitutions of the conserved residue His^{118} to Ala or to Glu [9] or to Gly [10] in *E. coli* EF-Tu also cause significant decreases in the affinity of EF-Tu for aa-tRNA. His^{118} is in the immediate structural neighbourhood of Leu^{120} and Gln^{124} in the interface of domains I and III. Tapio et al. [5] found that the EF-Tu mutant Aa [32] has almost an order of magnitude lower affinity for Phe-tRNA^{Phe} than wild-type EF-Tu. Sequence analysis [13] revealed that the tufAa mutation leads to a Glu^{378}Lys change in domain III, also in the interface between domains I and III. In summary, all these cases show that the details of the amino acid sequences of the interface between domains I and III are important for the stability of the ternary complex.

Perturbations of the binding of aminoacyl-tRNA to EF-Tu by amino acid substitutions in the interface between domains I and III cannot easily be explained by the recently determined structure of the ternary complex of Phe-tRNA·EF-Tu·GDPNP [16]. In that structure the binding sites for the tRNA on EF-Tu are (i) the exposed surface of domain III which binds the T stem of tRNA, (ii) the junction of all three domains which forms a pocket for the 5′-phosphate of tRNA, and (iii) a narrow cleft between domains I and II which binds the 3′ CCA-Phe end of tRNA. The surface of domain III which is in contact with the T stem of tRNA is not near the interface of domains I and III (Jens Nyborg, personal communication). However, we can envisage a number of

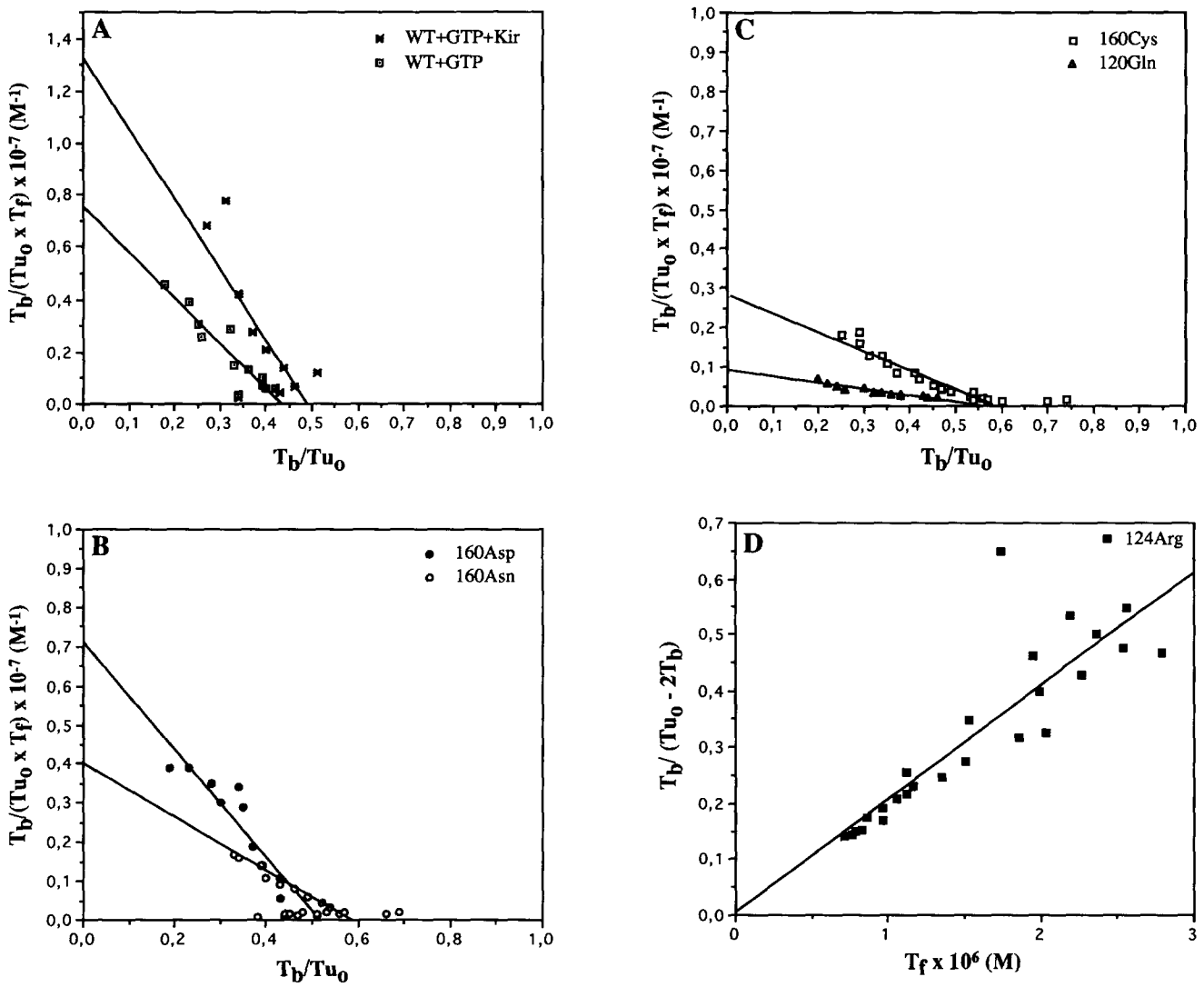

Fig. 3. (A–C) Scatchard plots to determine association constants (K_A) for the interaction between aa-tRNA and EF-Tu·GTP (section 2). T_b and T_f are bound and free, respectively, Phe-tRNA^{Phe} (see section 2 on how T_b and T_f are determined experimentally) and Tu_0 is the total concentration of active EF-Tu. The intercept at the y-axis provides K_A and the intercept at the x-axis gives how many molecules of Phe-tRNA^{Phe} that are protected by one molecule of active EF-Tu. All intercepts at the x-axis are similar and close to 0.5, indicating that it takes two molecules of EF-Tu to protect one molecule of aa-tRNA from deacylation under those conditions. (A) Wild-type EF-Tu·GTP with or without kirromycin (100 mM). (B) The EF-Tu·GTP variants ^{160}Asp, and ^{160}Asn. (C) The EF-Tu·GTP variants ^{160}Cys, and ^{120}Gln. (D) Association constant for the EF-Tu·GTP variant ^{124}Arg, determined by assuming that two molecules of EF-Tu·GTP protect one Phe-tRNA^{Phe} from deacylation, as in the other five cases. The slope of the line gives the K_A value.

models which could, in principle, account for the perturbations caused by the substitutions. One possibility is that the substitutions in the domain I-III interface change the configuration of EF-Tu, and that it is these larger scale structural alterations which slightly affect the relative spatial orientation of the three areas for the binding of aminoacyl-tRNA. A second possibility is that the substitutions affect the rather large conformational change which occurs when activating the GDP form of EF-Tu into its GTP form [1] in such a way as to shift the equilibrium towards the GDP form.

The observations that kirromycin resistance often is associated with reduced affinity between EF-Tu·GTP and aa-tRNA ([5,30], this work) suggest a causal relationship between resistance and affinity loss. To investigate this further we studied the strength of Phe-tRNA^{Phe} binding to wild-type EF-Tu·GTP in the presence of kirromycin and found it to be 2-fold higher than without the drug (Fig. 3A). Pingoud et al. [27,29] found a 2-fold reduction in the affinity of Tyr-tRNA^{Tyr} to EF-Tu·GTP by the action of kirromycin. Taken together these results demonstrate small but significant effects of kirromycin on the stability of the wild-type ternary complex. The contrasting claim by Abrahams et al. [33], that kirromycin reduces the binding of aa-tRNA to EF-Tu·GTP by 3 orders of magnitude, may be due to their having EF-Tu·GDP, rather than EF-Tu·GTP, in several of their assays. It is known that kirromycin enhances the GTPase activity of the ternary complex [17,33], so that the complex tends to fall apart on conversion of GTP to GDP on EF-Tu. To avoid this artifact special precautions must be taken. This was done in our experiments as well as in those of Pingoud et al. [27,29] by having a powerful energy regeneration system to keep the concentration of free GTP at a high level and the concentra-

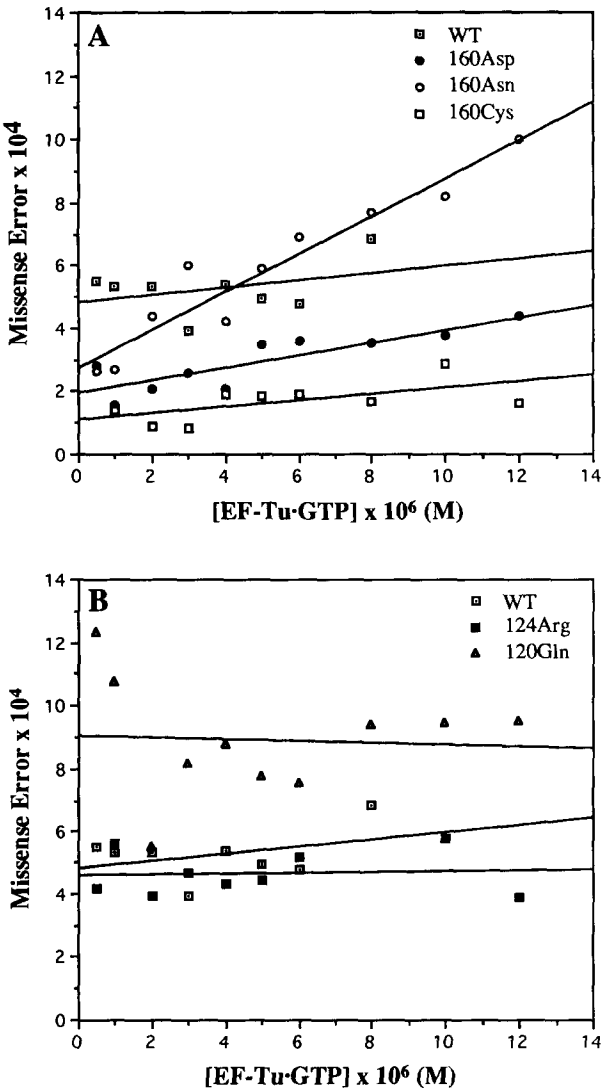

Fig. 4. Determination of the missense error frequency at different concentrations of EF-Tu·GTP for Leu-tRNA^{Leu} isoacceptors in bulk tRNA competing with Phe-tRNA^{Phe} for poly(U) programmed ribosomes (section 2). (A) Wild-type EF-Tu and the variants ^{160}Asp, ^{160}Asn and ^{160}Cys. (B) Wild-type EF-TU and the variants ^{124}Arg and ^{120}Gln.

tion of free GDP at a very low value [25]. In this way the ternary complex could be stably maintained, in spite of the kirromycin induced GTPase activity. In the gel assays used by Abrahams et al. [33] there was no GTP regeneration to prevent an irreversible, kirromycin induced, conversion of GTP to GDP in the ternary complex.

The fact that wild-type EF-Tu·GTP can bind aa-tRNA and kirromycin simultaneously makes it very unlikely that they have overlapping binding sites. The basis of the causal relationship between kirromycin resistance and reduced affinity for aa-tRNA may be the location of the mutations in the domain interface, a location which could plausibly affect the conformational switching of EF-Tu, reducing its affinity for both ligands. This interpretation would suggest that though mutations to kirromycin resistance map to both sides of the domain I-III interface [13], the actual binding site of kirromycin might be elsewhere, for example on domain I, but be influenced by the interdomain interactions.

To probe further the origin of kirromycin resistance, we measured the binding constants for the complex between Phe-tRNA^{Phe} and the kirromycin resistant EF-Tu mutants ^{120}Gln and ^{160}Cys and found these not to be influenced by kirromycin. One possibility is that these EF-Tu variants have such a low affinity for kirromycin, that binding is negligible up to drug concentrations of 10^{-4} M (section 2), and that the kirromycin resistance of these variants is due, at least partially, to their low affinity for the drug.

We measured a 200-fold lower association constant for the binding of Phe-tRNA^{Phe} to EF-Tu·GDP than to EF-Tu·GTP, in accordance with previous results [7,27,29]. At the same time, EF-Tu·GDP did not protect Phe-tRNA^{Phe} from spontaneous deacylation in the presence of kirromycin. Since EF-Tu·GDP·kirromycin can bind aminoacyl-tRNA, there must exist a ternary complex configuration that does not protect the aminoacyl bond [29]. This configuration cannot be identical with the ordinary ternary complex between EF-Tu·GTP and aa-tRNA, where there is good protection of aa-tRNA. From this we argue that the notion that kirromycin preserves EF-Tu in its GTP configuration as the ternary complex complex hits the A-site with concomitant GTP hydrolysis is an oversimplification: there must be a third structure of EF-Tu with very high affinity for the A-site (V. Dincbas and M. Ehrenberg, in preparation). It is, furthermore, quite possible that mutant EF-Tu's that do not bind kirromycin in their free state, are able to do so when they are in the A-site. In such a case a weakened binding between EF-Tu and aa-tRNA would make it easier for EF-Tu·GDP·kirromycin complexes to leave the A-site, and this may be the reason why kirromycin resistance is frequently associated with low affinity for aa-tRNA binding to EF-Tu.

Measurements of missense errors for the different kirromycin resistant EF-Tu mutants reveal differential effects on their binding to different aminoacyl-tRNAs, as well as perturbations in their interaction with the ribosome. There is evidence for increased (^{120}Gln), for unchanged (^{124}Arg) and for decreased error levels (^{160}Cys) (Fig. 4A,B). In these three cases (and also for ^{160}Asp) missense errors do not vary much with the concentration of EF-Tu·GTP, indicating that the affinities of Leu-tRNA^{Leu} and Phe-tRNA^{Phe} to EF-Tu·GTP are quite similar. In contrast, for ^{160}Asn missense errors increase significantly with increasing EF-Tu concentrations (Fig. 4A). This indicates that for this factor mutant the binding of Leu-tRNA^{Leu} to EF-Tu·GTP is significantly weaker than the binding of Phe-tRNA^{Phe}. In the case of the mutant ^{160}Asn (Fig. 4A) the error level is lower than wild-type at low, and higher than wild-type at high, EF-Tu concentrations. This shows, firstly, that Leu-tRNA^{Leu} has a significantly lower affinity to the ^{160}Asn variant than does Phe-tRNA^{Phe}. The amino acid change has in this case reduced the affinity of Phe-tRNA^{Phe} for EF-Tu about 2-fold (Table 1) but the affinity of Leu-tRNA^{Leu} must be reduced much further. It shows, secondly, that for this EF-Tu variant a Leu-tRNA^{Leu} containing ternary complex competes better with a Phe-tRNA^{Phe} ternary complex for the ribosomal A-site than when EF-Tu is wild-type. The enhanced missense errors at excess EF-Tu concentrations may reflect a general stabilization of the binding between EF-Tu and the A-site by the Tyr→Asn change at position 160 (cf. [34,35]). A similar type of argument suggests that the Tyr→Cys change at the same position leads to a universal destabilization of ternary complex binding to the A-site caus-

ing reduced (Fig. 4A), rather than enhanced, missense levels.

One mutant, $Tyr^{160}Cys$, also has a significantly enhanced exchange rate of GDP on EF-Tu. Interestingly, the substitution $His^{118}Gly$ also alters the interaction between guanine nucleotide and EF-Tu, decreasing EF-Tu's intrinsic GTPase activity in the absence, but increasing it in the presence of aa-tRNA [10]. Thus, substitutions in the domain interface, many of which apparently decrease the affinity of aa-tRNA for EF-Tu, are in some cases also associated with alterations in the interaction between EF-Tu and the bound guanine nucleotide. Current structural information [1,2,31] does not indicate any direct interaction between these residues and the guanine nucleotide, suggesting that these effects are indirect.

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