Mutant species of EF-Tu, altered at position 375, exhibit a reduced affinity for aminoacylated transfer-RNAs

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The interaction between EF-Tu · GTP and aminoacyl-tRNA is shown to be influenced by mutations at site 375 of this three-domain protein. Site 375 is located in domain II near the interface with domain I [(1984) EMBO J. 3, 113–120]. Replacement of the alanine at this site by a threonine or valine residue results in lower binding constants with Phe-tRNA and Tyr-tRNA, as was evaluated by the hydrolysis protection technique. The data are discussed in the light of what is known about the three-dimensional structure of the protein and its interaction sites with aminoacyl-tRNA.

Elongation factor EF-Tu *Protein-RNA interaction* *Binding constant* *Protein biosynthesis* *Multi-domain protein* *Aminoacyl-tRNA hydrolysis*

1. INTRODUCTION

Elongation factor EF-Tu is a multifunctional protein involved in protein biosynthesis [1], viral RNA replication [2], and regulation of its own expression [3]. Wild-type EF-Tu from *Escherichia coli* is coded for by two genes with almost identical gene products [4], and is here denoted as EF-Tu(As + Bs).

Several kirromycin-resistant *E. coli* strains have been isolated that express only one of the two EF-Tu encoding genes [3]. EF-Tu from two of these mutant strains has its alanine residue at position 375 replaced by another amino acid, i.e., threonine (EF-Tu(Ar) from strain LBE2045) or valine (EF-Tu(A) from strain D2216) [5,6]. Since Ala-375 is located in a region which is probably involved in the control of various conformational states of EF-Tu [6], it was of considerable interest to study the consequences of such a substitution for the function of EF-Tu. Previously the GTPase activity and the GDP and GTP rate and equilibrium constants have been determined [7]. Here we have studied the effect of the mutation on the interaction of EF-Tu · GTP with aminoacyl-tRNA. The binding constant has been determined with the procedure of Pingoud et al. [8], which is based on the protection provided by EF-Tu against hydrolysis of the aminoacyl ester bond in the ternary complex aminoacyl-tRNA–EF-Tu · GTP.

2. MATERIALS AND METHODS

Wild-type cells and mutant cells of strain LBE2045 of *E. coli* were grown in a 14-l fermentation apparatus and the cells were harvested in their mid-log phase with a continuous-flow rotor. The bacteria were stored at −20°C until use. Elongation factor EF-Tu was isolated from bacterial cell extracts, obtained after treatment of the cells with lysozyme and sonication, using ion-exchange chromatography and gel filtration [9]. The purity of EF-Tu was more than 95% as judged by SDS-polyacrylamide gel electrophoresis. The activity of EF-Tu was between 70 and 90% of its theoretical value as was calculated from the GDP exchange

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assay [8]. No contamination of the preparation by non-specific GTPases could be detected by a GTPase activity assay. Mutant EF-Tu from strain D2216 was isolated in an analogous way.

Charging of tRNAs with their cognate amino acids was performed as described before [10]. The specific activity of [^{14}C]Tyr-tRNA^{Tyr} (*E. coli*) was 451 cpm/pmol and that of [^{14}C]Phe-tRNA^{Phe} (yeast) was 149 cpm/pmol. These aa-tRNAs were stored at −20°C.

In the hydrolysis protection experiment EF-Tu·GDP and aa-tRNA were incubated at 25°C in a pH 7.4 buffer containing 75 mM Tris-HCl, 75 mM NH_4Cl, 15 mM $MgCl_2$, 7.5 mM dithioerythritol, 2.2 mM phospho*enol*pyruvate, 0.1 mM GTP and 0.15 mg/ml pyruvate kinase (Boehringer). At times between 0 and 3 h aliquots of 20 μl were pipetted onto Whatman 3 MM filter disks, which were then directly immersed in a cold 10% (w/v) trichloroacetic acid solution and after 10 min washed with a 5% trichloroacetic acid solution and an ether/ethanol mixture. The dried filters were counted in the scintillation counter after addition of scintillation liquid.

For the calculation of the aminoacyl-tRNA–EF-Tu·GTP binding constants the following 3 reactions have to be considered [10]:

$$\text{aa-tRNA} \xrightarrow{k_f} \text{amino acid} + \text{tRNA} \quad (1)$$

$$\text{aa-tRNA–EF-Tu·GTP} \xrightarrow{k_b} \text{amino acid} + \text{tRNA} + \text{EF-Tu·GTP} \quad (2)$$

$$\text{aa-tRNA} + \text{EF-Tu·GTP} \overset{K_c}{\rightleftharpoons} \text{aa-tRNA–EF-Tu·GTP} \quad (3)$$

Here k_f is the rate constant for deacylation of free aa-tRNA, k_b is the rate constant for deacylation of aa-tRNA bound to EF-Tu·GTP and K_c is the equilibrium constant of ternary complex formation between EF-Tu·GTP and aa-tRNA. This binding constant is given by:

$$K_c = \frac{S(b)}{S(f)E(f)} \quad (4)$$

Here $S(f)$ and $S(b)$ are the concentrations of free and complex bound aa-tRNA, and $E(f)$ is the concentration of unbound EF-Tu·GTP. The deacylation reactions 1 and 2 are treated as (pseudo) first-order reactions of which k_f and k_b are the respective reaction rate constants. The initial rate of hydrolysis v_I was experimentally found to be linear with the total concentration of aa-tRNA:

$$v_I = k_I S^o \quad (5)$$

Here S^o is the total concentration of free and bound aa-tRNA at the beginning of the experiment. A complete hydrolysis protection experiment includes a series of partial experiments, all with the same starting concentration of total aa-tRNA (S^o) but with different total concentrations of EF-Tu·GTP (E). Each of these partial experiments gives a different value for k_I, due to the presence of different concentrations of EF-Tu. The initial rate constants of hydrolysis k_I can be calculated most conveniently from log-linear plots of S^t/S^o vs time [11], where S^t is the total concentration of aa-tRNA at a given time. An aa-tRNA hydrolysis experiment in the absence of EF-Tu renders a value for k_f. An estimate of the value of k_b can be obtained graphically from the plot of $1/(k_f - k_I)$ vs $1/E$ [11].

Combination of eqns 1–5 renders the following equation:

$$\frac{q}{(1-q)} \cdot \frac{1}{E} = nK_c - K_c \cdot \frac{qS^o}{E};$$

$$q = (k_I - k_b)/(k_f - k_b) \quad (6)$$

If q is determined for different values of E, then K_c and n can be evaluated from a linear plot of qS^o/E vs $q/[(1-q)E]$. EF-Tu preparations are usually less than 100% active in ternary complex formation; this is corrected for by the factor n, reflecting the mole fraction of EF-Tu molecules that participate in the binding process. The linear plot gives a slope of $-K_c$ and an intercept n with the x-axis. The advantage of this approach is that the binding constants evaluated in this way are truly independent of the percentage of activity of the EF-Tu preparation.

3. RESULTS

The method used for the determination of the binding constant of EF-Tu·GTP and aa-tRNA is based on the protection against hydrolysis of the aminoacyl ester bond provided by EF-Tu in the aminoacyl-tRNA–EF-Tu·GTP ternary complex.

The hydrolysis data of Phe-tRNA (fig.1) and Tyr-tRNA (fig.2) demonstrate this protection. Furthermore, they show that EF-Tu(As + Bs) (figs 1a,2a) is more efficient in this respect than EF-Tu(Ar) (figs 1b,2b).

A series of protection experiments was performed with several kinds of aa-tRNA and EF-Tu. The logarithm of the concentration of aa-tRNA was plotted as a function of time and the k_1 and S^o values were calculated. An example of such a plot is given in fig.3. The k_b value, necessary to calculate q, was estimated from a plot of $1/(k_f - k_1)$ vs $1/E$ (fig.4). Although the value of 10×10^{-5} min^{-1} for k_b thus estimated was rather unreliable, it will not affect the value of q in a significant way as $k_b \ll k_f$.

With the values of the rate constants (k_1) for the hydrolysis reaction, the initial concentrations of aa-tRNA (S^o) and the total concentration of EF-Tu (E), the hydrolysis data can now be transformed into a linear plot according to eqn 6. An example of such a linearized plot is given in fig.5 for the hydrolysis protection of Phe-tRNA by wild-type and mutant EF-Tu. From the slope of the straight line K_c was calculated and the intercept with the x-axis yielded the fraction n of EF-Tu that was participating in ternary complex formation. The fact that a considerably smaller fraction of EF-Tu(D2216) was found to participate in ternary complex formation as compared with EF-Tu(Ar) or with wild-type EF-Tu reflects the greater instability of this particular EF-Tu. From the slopes it can be seen directly that the affinities of Phe-tRNA for wild-type and mutant EF-Tu differ significantly. Table 1 summarizes the results, showing that the binding constant of Phe-tRNA is about a factor of 3 lower for EF-Tu(Ar) and EF-Tu(D2216) than that for EF-Tu(As + Bs). In the case of Tyr-tRNA, binding constants were found about a factor of 6 lower for EF-Tu(Ar) than for EF-Tu(As + Bs).

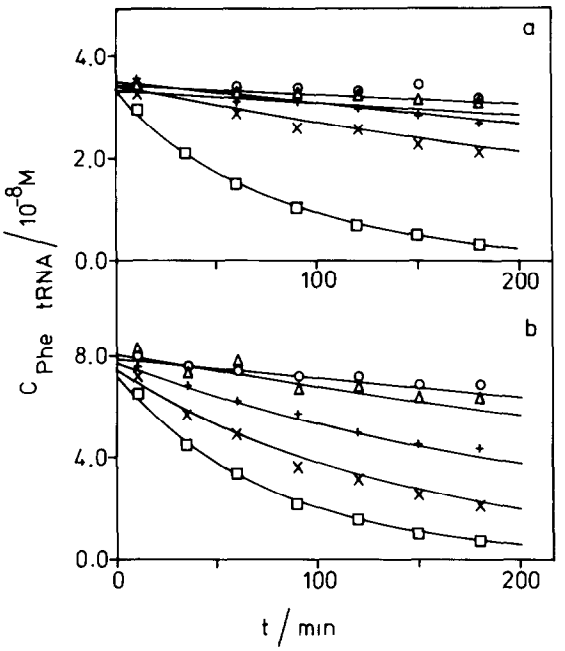

Fig.1. Hydrolysis protection of Phe-tRNA by EF-Tu·GTP at 25°C. (a) EF-Tu(As + Bs)·GTP at concentrations of 0.78 (○), 0.52 (△), 0.28 (+), 0.13 (×) and 0.00 (□) μM. (b) EF-Tu(Ar)·GTP at concentrations of 2.37 (○), 1.32 (△), 0.53 (+), 0.20 (×) and 0.00 (□) μM.

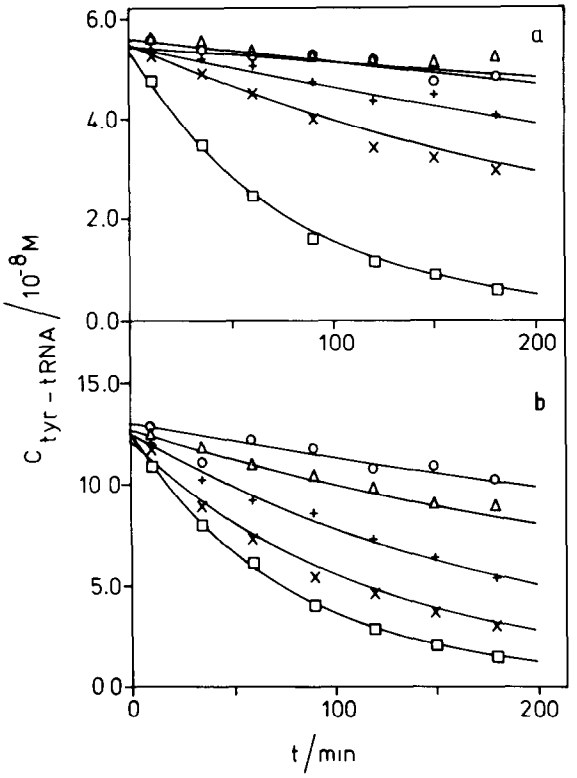

Fig.2. Hydrolysis protection of Tyr-tRNA by EF-Tu·GTP at 25°C. (a) EF-Tu(As + Bs)·GTP at concentrations of 0.92 (○), 0.78 (△), 0.26 (+), 0.13 (×) and 0.00 (□) μM. (b) EF-Tu(Ar)·GTP at concentrations of 2.36 (○), 1.32 (△), 0.53 (+), 0.20 (×) and 0.00 (□) μM.

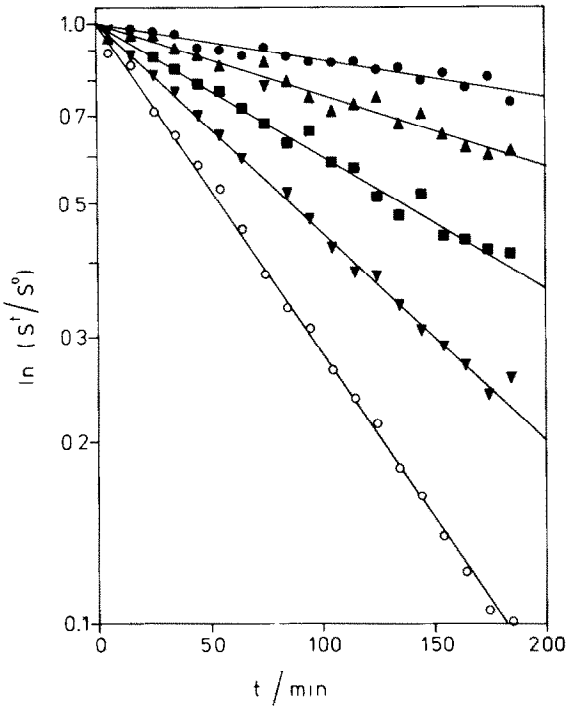

Fig.3. Hydrolysis protection of Phe-tRNA by EF-Tu(Ar)·GTP. Semilogarithmic plot for the hydrolysis of 0.20 μM Phe-tRNA (S^0) in the presence of 0.00 (○), 0.20 (▲), 0.41 (■), 0.82 (▼) and 1.64 (●) μM EF-Tu(Ar)·GTP.

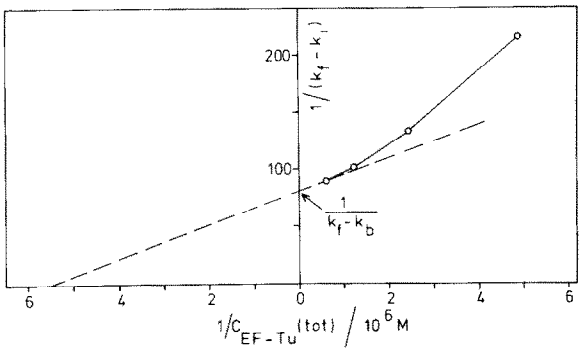

Fig.4. Graphical estimation of the rate of hydrolysis of Phe-tRNA in the ternary complex with EF-Tu(Ar)·GTP [11].

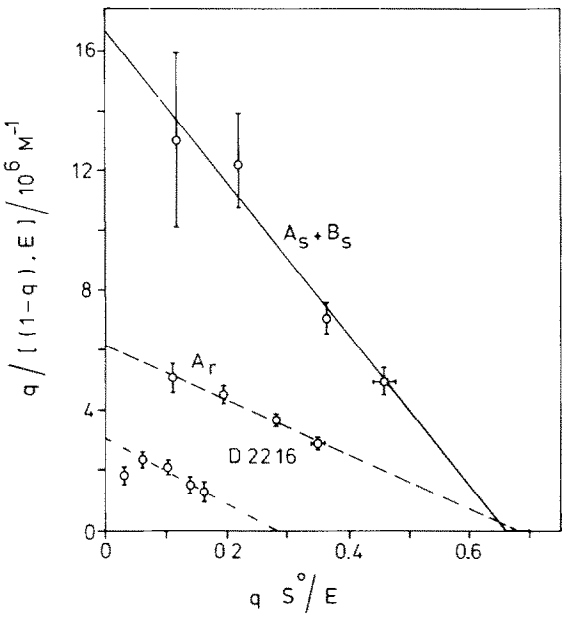

Fig.5. Linear plot for the binding of Phe-tRNA to EF-Tu(As+Bs)·GTP, EF-Tu(Ar)·GTP and to EF-Tu·GTP from strain D2216 at 25°C. See eqn 6.

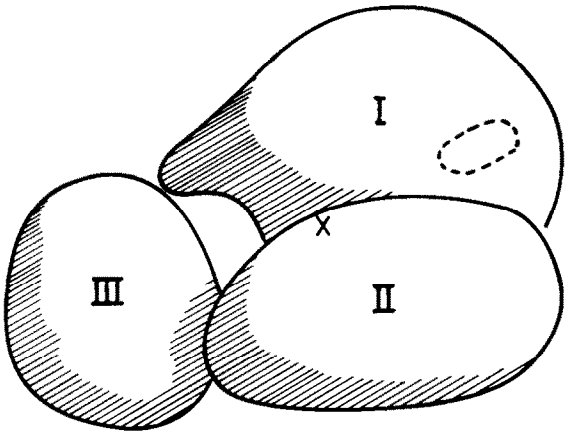

Fig.6. Three-dimensional representation of the overall shapes of the domains of EF-Tu [6]. The position of the nucleotide binding site in domain I is shown by the dashed region. The approximate location of the mutation site is indicated by ×.

The binding constant of EF-Tu(As+Bs)·GTP for Phe-tRNA differs from the published value of $(50 \pm 20) \times 10^6$ M^{-1} [10]. The latter, however, was obtained not taking into account that possibly a fraction of EF-Tu does not participate in the binding. Analysis of our data in the same way as in [10] yielded values for K_c of 39–44 $\times$ 10^6 M^{-1}. The discrepancy between our value of the binding con-

Table 1

Binding constants (± 1 SD) for ternary complex formation between aminoacyl-tRNA and different species of elongation factor EF-Tu·GTP

	K_c (10^6 M^{-1})		
aa-tRNA	EF-Tu(As + Bs)	EF-Tu(Ar)	EF-Tu(D2216)
Phe-tRNA	25 ± 4 (66%)	9.2 ± 0.6 (68%)	10.8 ± 1.1 (28%)
Tyr-tRNA	44 ± 10 (77%)	7.8 ± 0.4 (57%)	–

The fractions of EF-Tu molecules participating in the binding equilibrium are given in parentheses. (–) Experiment not performed

stant of EF-Tu(As + Bs)·GTP for Tyr-tRNA and the previously determined one of $(7 \pm 2) \times 10^6$ M^{-1} cannot be explained in this way, as the latter value is lower compared to the former. Different batches of Tyr-tRNA and the radioactive amino acid are probably responsible for the difference.

The ratio of binding constants of EF-Tu-As + Bs)·GTP for Phe-tRNA and Tyr-tRNA is 0.6. This ratio is in good agreement with a ratio of 0.7 as calculated from the competition form of the ribonuclease resistance assay performed at 4°C [12].

4. DISCUSSION

Our data demonstrate that substitution of Ala-375 by Thr or Val reduces the affinity of EF-Tu·GTP for aa-tRNA (cf. table 1). X-ray diffraction studies have traced residue 375 on top of domain II, not far from the interface with domain I (fig.6) [13].

In the recognition of EF-Tu·GTP, the 3′-terminus of aa-tRNA plays a key role [14–17]. Recently, tRNA oxidized with periodate, positioned at the ribosomal A-site in the presence of EF-Tu·GTP under conditions that the ribosomal P-site was occupied by *N*-acetylaminoacyl-tRNA, was found cross-linked to the ϵ-NH_2 group of Lys-237 [18]. The latter residue is located at α-helix VI of domain I [13]. This experiment locates the 3′-end of tRNA in the ternary complex in close proximity to the cleft between β-strand 1 and α-helix VI. Secondly, binding of aa-tRNA and 3′-terminal fragments thereof to EF-Tu·GTP protects Cys-81 at the end of β-strand 2 against modification with thiol agents [14]. Finally, ϵ-*N*-bromoacetyllysyl-tRNA has been cross-linked to His-66 on β-strand 1 [19]. Apparently, a major site of interaction with aa-tRNA is on domain I, rather distant from Ala-375. It is conceivable, however, that other sites of interaction exist and that replacement of Ala-375 might cause a direct steric hindrance at these sites. Alternatively, it has been suggested earlier [6] that a mutation so close to the interface of domains I and II, could influence their relative positions, preventing optimal aa-tRNA binding. Since ternary complex formation presumably imposes a conformational change on EF-Tu·GTP, it is conceivable and in agreement with the lower binding constants, that mutant EF-Tu·GTP needs more energy to undergo such a conformational change than its wild-type counterpart.

Up to the present it is not clear whether the altered affinity for aa-tRNA binding is due to either steric hindrance by Thr or Val around position 375 or to an allosteric effect of the mutation.

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