Mutational analysis of *Escherichia coli* elongation factor Tu in search of a role for the N-terminal region


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We have mutated lysine 2 and arginine 7 in elongation factor Tu from *Escherichia coli* separately either to alanine or glutamic acid. The aim of the work was to reveal the possible interactions between the conserved N-terminal part of the molecule, which is rich in basic residues and aminoacyl-tRNA. The enzymatic characterization, comprising GDP and GTP temperature stability assays and measurement of nucleotide dissociation and association rate constants, GTPase activity and aminoacyl-tRNA binding, shows that position 2 is not involved in aminoacyl-tRNA binding, while position 7 is necessary to accomplish this activity. Furthermore, arginine 7 seems to play a role in regulating the binding of GTP. The three-dimensional structure of the ternary complex, EF-Tu:GTP:Phe-tRNA, involving *Thermus aquaticus* EF-Tu and yeast Phe-tRNA, shows that Arg 7 is in a position which permits salt bridge formation with Asp 284, thus binding the N-terminus tightly to domain 2. We propose that this interaction is needed for aminoacyl-tRNA binding, and also for completing the structural rearrangement, which takes place when the factor switches from its GDP to its GTP form.

Keywords: aminoacyl-tRNA binding/basic residues/elongation factor Tu/mutational analysis/structural rearrangement

Introduction

Elongation factor Tu (EF-Tu) belongs to the group of GDP/GTP-binding proteins, which are defined by their ability to bind the guanine nucleotide derivatives, guanosine diphosphate (GDP) or triphosphate (GTP). The active state or ‘on’-state corresponds to the GTP form, while the inactive state or ‘off’-state corresponds to the GDP form.

In its active form, EF-Tu:GTP binds aminoacyl-tRNA (aa-tRNA) and accomplishes its main function to carry aa-tRNA to the translating ribosome, where the aa-tRNA will recognize the sequence complementary to its anticodon and add another amino acid to the nascent polypeptide chain. There is only one EF-Tu molecule for the many different structures of aa-tRNA. Therefore, one role of EF-Tu is probably to ensure that all the different aa-tRNA molecules can be presented to the ribosomes in almost the same shape (Barciszewski et al., 1994). EF-Tu also has an active role in proof-reading, helping to discern between near-cognate aa-tRNA and cognate aa-tRNA (Thompson, 1988). Furthermore, EF-Tu is one of the subunits of the replicase complex of bacteriophage Qβ (Blumenthal and Carmichael, 1979).

Under physiological conditions, EF-Tu is normally bound to aa-tRNA, but to do so EF-Tu has to be in the GTP form. However, the affinity for GDP is 500 times higher than that for GTP (Kd = 1 and 500 nM, respectively; Parmeggiani and Sander, 1981). There are several factors that help to overcome this. The GTP concentration in the cell exceeds the concentration of GDP 10-fold. In addition, the elongation factor Ts (EF-Ts) and aa-tRNA drive the reaction towards the desired complex. EF-Ts promotes nucleotide exchange and aa-tRNA binds EF-Tu, thus sequestering the GTP form.

The structures of EF-Tu in complexes with GDP and GTP from different species are known (Kjeldgaard and Nyborg, 1992; Berchtold et al., 1993; Kjeldgaard et al., 1993; Abel et al., 1996; Polekhina et al., 1996). From these structures, many details are known concerning nucleotide binding, magnesium binding and the overall switch of the structure from the relaxed GDP form to the more constrained GTP form. However, it was not until recently that the structures of the EF-Tu in complexes with aa-tRNA (Nissen et al., 1995) and elongation factor Ts (Kawashima et al., 1996) were solved. These structures have enabled us to understand more about the molecular details of RNA–protein and protein–protein interactions.

The N-terminal region of the protein is of special interest since it still is not very well resolved owing to its extended conformation. The importance of several residues at the N-terminal region of EF-Tu in binding aa-tRNA has been pointed out in several studies based on chemical modifications of basic residues (Kraal and Hartley, 1978; Marschel and Bodley, 1980; Antonsson and Leberman, 1984). These studies indicate that lysines 2 and 4 and arginine 7 could be involved in aa-tRNA binding. Two of these residues, lysine 2 and arginine 7, were the subject of this study.

When comparing the sequences of EF-Tu from different organisms (Zeeff, 1994), Lys 2 turns out to be present in more than 50% of the organisms while the rest contain mainly an Arg in this position, i.e. the charge is conserved. In the case of Arg 7, more than 98% of the sequences contain an Arg in this position. In the eukaryotic version of EF-Tu, EF-1α, Arg 7 is replaced by an almost 100% conserved Lys. To a first approximation, the abundance of positive charges in the N-terminal region (Lys 2, Lys 4, Arg 7 and Lys 9), suggests a possible role in binding to the negative phosphate backbone of the aa-tRNA, which is considered to be one of the common features of aa-tRNA identified by EF-Tu.

With this background, Lys 2 and Arg 7 were both mutated to either alanine or glutamic acid, resulting in four single-point mutations named TuLys 2 Ala, TuLys 2 Glu, TuArg 7 Ala and TuArg 7 Glu. We have characterised these mutants biochemically and from the results we propose a role for Lys 2 and Arg 7.

Materials and methods

The Sequenase 2.0 kit, BamHI and the Sculptor in vitro mutagenesis system were purchased from Amersham/USB. The oligodeoxynucleotides were obtained from Genosys Custom Biopolymers. T4 ligase and Smal were purchased from New York University Press.
Fig. 1. Dissociation of (a) EF-Tu:GDP and (b) EF-Tu:GTP complexes at 0°C. \( c_t \) and \( c_0 \) correspond to the concentration of the complexes at time \( t \) and the initial concentration, respectively. The correlation coefficients for the plots are above 0.99 and 0.96 for the GDP and GTP forms, respectively. (●) wild type; (■) TuLys2Ala; (●) TuLys2Glu; (▲) TuArg7Ala; (▼) TuArg7Glu. The dissociation rate constants calculated from the plots are given in Table I.

Fig. 2. Association of EF-Tu and (a) GDP and (b) GTP at 0°C. GDP\(_0\) and GTP\(_0\) are the initial concentrations of the nucleotides. Tu\(_0\) is the initial concentration of EF-Tu. The concentration of the complexes EF-Tu:GDP/GTP at different times are denoted by \( x \). Correlation coefficients are above 0.94 and 0.92 for the GDP and GTP forms, respectively. (●) wild type; (■) TuLys2Ala; (●) TuLys2Glu; (▲) TuArg7Ala; (▼) TuArg7Glu. The association rate constants calculated from the plots are given in Table I.

England Biolabs. Alkaline phosphatase was provided by Boehringer. The Escherichia coli strain JM109 was obtained from Stratagene (San Diego, CA). The pGEX vector was kindly provided by Naomi Halachmi (Technion, Haifa). Factor Xa was obtained from Denzyme Aps (Århus Science Park, Denmark), tRNA\(^{Phe}\) (yeast) and glutathione agarose were purchased from Sigma. Cellulose-acetate filters for GDP binding assays were supplied by Gelman Sciences and for the GTP form of EF-Tu by Schleicher and Schuell. \[^3H\]GDP, \[^3H\]GTP, \[^14C\]Phe and \[^\gamma\]\(^32\)P\]GTP were obtained from Amersham/USB. Kirromycin was kindly provided by Gist-Brocades (Delft, The Netherlands). Centricon 30 and Centriprep 30 were both obtained from Amicon.

Constructions of mutants

Site-directed mutagenesis was performed following the phosphorothioate method according to Taylor (Taylor et al., 1985). Single-stranded M13mp11 containing a recognition site for the protease factor Xa followed by the \( \text{tufA} \) gene encoding EF-Tu was used as a template (Knudsen et al., 1992) and the oligonucleotides 5’-GGTTTCTTGAGAAACTTTTTG-3’, 5’-GGTCTTCTGAAGAAATTTG-3’, 5’-GAAAAATTT-GAAGCAGAAAAACC-3’ and 5’-GAAAAATTGGAAGACAAAACC-3’ were used as primers for the mutations TuLys2Ala, TuLys2Glu, TuArg7Ala and TuArg7Glu, respectively.

The FX\(\text{tufA} \) [TuLys2Ala, TuLys2Glu, TuArg7Ala and TuArg7Glu] fragments were excised by digestion with BamH\(\text{I} \) and cloned into the pGEX1 expression vector (Smith and Johnson, 1988). The \( \text{E.coli} \) strain JM109 was used as host.

The mutations were confirmed by single- and double-stranded dideoxy sequencing (Sanger et al., 1977; Sambrook et al., 1989).

Expression and purification

We followed the procedure described by Knudsen et al. (1992) with the following exception. The cells were opened by treatment with lysozyme as follows: the cells were thawed on ice and resuspended in 2.5 ml/g cell ice-cold buffer S (50 mM Tris–HCl, pH 7.6, at 4°C, 10 mM MgCl\(_2\), 15 µM GDP, 1% Triton X-100); 0.5 mg of lysozyme was added per gram of cells and the solution was left for at least 5 min at 20°C with very slow stirring, then 0.1 µl of 2% sodium deoxycholate and 250 U of DNase were added per gram of cells.
Table I. Nucleotide dissociation and association rate constants, $k_{1}$ and $k_{-1}$, determined at 0°C

<table>
<thead>
<tr>
<th>EF-Tu species</th>
<th>GDP $k_{1} \times 10^{4}$ (M$^{-1}$ s$^{-1}$)</th>
<th>GDP $k_{-1}$ (s$^{-1}$)</th>
<th>GTP $k_{1} \times 10^{4}$ (M$^{-1}$ s$^{-1}$)</th>
<th>GTP $k_{-1}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type EF-Tu</td>
<td>21.6 ± 1.14</td>
<td>2.85 ± 0.31</td>
<td>1.28 ± 0.2</td>
<td>48.1 ± 8.0</td>
</tr>
<tr>
<td>TuLys2Ala</td>
<td>19.3 ± 2.82</td>
<td>2.66 ± 0.27</td>
<td>1.08 ± 0.16</td>
<td>40.7 ± 5.8</td>
</tr>
<tr>
<td>TuLys2Glu</td>
<td>21.2 ± 1.75</td>
<td>2.74 ± 0.23</td>
<td>0.67 ± 0.08</td>
<td>39.5 ± 3.2</td>
</tr>
<tr>
<td>TuArg7Ala</td>
<td>16.9 ± 1.41</td>
<td>2.91 ± 0.23</td>
<td>0.75 ± 0.11</td>
<td>68.5 ± 6.0</td>
</tr>
<tr>
<td>TuArg7Glu</td>
<td>0.83 ± 0.85</td>
<td>2.13 ± 0.23</td>
<td>0.13 ± 0.02</td>
<td>64.5 ± 2.7</td>
</tr>
<tr>
<td>Fasano et al. (1978)</td>
<td>26</td>
<td>2.3</td>
<td>1.0</td>
<td>59</td>
</tr>
<tr>
<td>Arai et al. (1974)</td>
<td>31</td>
<td>3.4</td>
<td>11.0</td>
<td>500</td>
</tr>
</tbody>
</table>

The apparent dissociation and association rate constants, $k_{1}$ and $k_{-1}$, for EF-Tu:GDP and EF-Tu:GTP were studied by the nitrocellulose filter procedure (Arai et al., 1974; Fasano et al., 1978). The values were calculated from the plots in Figures 1 and 2. Correlation coefficients were always $>$0.92.

Table II. Dissociation constants, $K_d$, for GDP and GTP

<table>
<thead>
<tr>
<th>EF-Tu species</th>
<th>$K_d$(GDP) (nM)</th>
<th>$K_d$(GTP) (nM)</th>
<th>$K_d$(GTP)/$K_d$(GDP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type EF-Tu</td>
<td>1.32</td>
<td>375</td>
<td>286</td>
</tr>
<tr>
<td>TuLys2Ala</td>
<td>1.38</td>
<td>377</td>
<td>273</td>
</tr>
<tr>
<td>TuLys2Glu</td>
<td>1.29</td>
<td>500</td>
<td>457</td>
</tr>
<tr>
<td>TuArg7Ala</td>
<td>1.72</td>
<td>913</td>
<td>530</td>
</tr>
<tr>
<td>TuArg7Glu</td>
<td>25.66</td>
<td>4962</td>
<td>193</td>
</tr>
<tr>
<td>Fasano et al. (1978)</td>
<td>0.9</td>
<td>590</td>
<td>656</td>
</tr>
<tr>
<td>Arai et al. (1974)</td>
<td>1.1</td>
<td>470</td>
<td>427</td>
</tr>
</tbody>
</table>

The dissociation constants are calculated as $k_d/k_{-1}$ for EF-Tu:GDP and EF-Tu:GTP at 0°C (Table I).

Table III. GTPase activity parameters determined at 30°C

<table>
<thead>
<tr>
<th>EF-Tu species</th>
<th>$k_m$ (µM)</th>
<th>$k_{cat} \times 10^6$ (s$^{-1}$)</th>
<th>$k_{cat}/k_m$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type EF-Tu</td>
<td>2.31</td>
<td>432</td>
<td>187</td>
</tr>
<tr>
<td>TuLys2Ala</td>
<td>1.28</td>
<td>488</td>
<td>381</td>
</tr>
<tr>
<td>TuLys2Glu</td>
<td>2.97</td>
<td>983</td>
<td>331</td>
</tr>
<tr>
<td>TuArg7Ala</td>
<td>1.52</td>
<td>344</td>
<td>226</td>
</tr>
<tr>
<td>TuArg7Glu</td>
<td>1.31</td>
<td>405</td>
<td>309</td>
</tr>
</tbody>
</table>

The concentration of [$^{32}$P]GTP was in the range 0.75–40 µM (400–2000 d.p.m./pmol) in the case of wild-type EF-Tu and 0.75–20 µM for the mutants. The EF-Tu concentration was kept constant at 0.6 µM for all of the EF-Tu species except TuLys2Gln, for which the assay was carried out at 1 µM EF-Tu. $K_m$ and $k_{cat}$ are calculated from Hanes plots with correlation coefficients $>0.91$.

The concentration of protein was measured with a modified version of the Bradford method (Sedmark and Grossberg, 1977) using bovine serum albumin as a standard.

EF-Tu wild type was purified in the same way to serve as a suitable reference.

Protein activity

The concentration of active protein was determined by maximum GDP exchange as described (Miller and Weisbach, 1977). 5 µM [$^{3}$H]GDP (1314 d.p.m./pmol) was incubated with 0.5 µM EF-Tu for 20 min at 30°C in binding buffer (50 mM Tris–HCl, pH 7.6, at 4°C, 100 mM NH$_4$Cl, 50 mM KCl, 10 mM MgCl$_2$, 1 mM DTT). The samples were then placed on ice for 10 min and finally spotted on nitrocellulose filters previously soaked in washing buffer (10 mM Tris–HCl, pH 7.6, at 4°C, 10 mM MgCl$_2$, 10 mM NH$_4$Cl). The filters were washed with 3×1 ml of washing buffer. The complex [$^{3}$H]GDP:EF-Tu bound to the filters was measured in a scintillation counter.

Table IV. Stimulation of the GTPase activity by kirromycin

<table>
<thead>
<tr>
<th>EF-Tu species</th>
<th>Velocity, $v$, in the absence of kirromycin (s$^{-1} \times 10^6$)</th>
<th>Velocity, $v$, in the presence of kirromycin (s$^{-1} \times 10^6$)</th>
<th>Relative effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type EF-Tu</td>
<td>190 ± 5</td>
<td>2137 ± 129</td>
<td>11.24</td>
</tr>
<tr>
<td>TuLys2Ala</td>
<td>142 ± 12</td>
<td>1278 ± 126</td>
<td>9.00</td>
</tr>
<tr>
<td>TuLys2Glu</td>
<td>477 ± 28</td>
<td>4559 ± 261</td>
<td>9.55</td>
</tr>
<tr>
<td>TuArg7Ala</td>
<td>142 ± 20</td>
<td>1626 ± 120</td>
<td>11.45</td>
</tr>
<tr>
<td>TuArg7Glu</td>
<td>153 ± 17</td>
<td>1525 ± 155</td>
<td>9.97</td>
</tr>
</tbody>
</table>

The GTPase assay contained 50 µM kirromycin and 0.3 µM EF-Tu. The [$^{32}$P]GTP concentration corresponds to the $K_m$ determined for each protein. The relative effect is calculated as ($v$ with kirromycin)/($v$ without kirromycin).

Table V. Apparent dissociation rate constant, $k_1$, and half-life, $T_1/2$, for the ternary complex, EF-Tu:GTP:Phe-tRNA$^{Phe}$

<table>
<thead>
<tr>
<th>EF-Tu species</th>
<th>$k_1 \times 10^{-3}$ (s$^{-1}$)</th>
<th>$T_1/2$ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type EF-Tu</td>
<td>1.5 ± 0.1</td>
<td>110 ± 23</td>
</tr>
<tr>
<td>TuLys2Ala</td>
<td>4.0 ± 0.29</td>
<td>72 ± 10</td>
</tr>
<tr>
<td>TuLys2Glu</td>
<td>3.4 ± 0.37</td>
<td>79 ± 11</td>
</tr>
<tr>
<td>TuArg7Ala</td>
<td>40.6 ± 6.0</td>
<td>37 ± 1.5</td>
</tr>
<tr>
<td>TuArg7Glu</td>
<td>45.9 ± 10</td>
<td>27 ± 3.0</td>
</tr>
</tbody>
</table>

Free aa-tRNA$^{Phe}$

Louie and Jurnak (1985) | 1.60 | – |
Andersen and Wiborg (1994) | 0.21 | 391 |
Kjærgaard et al. (1995) | 0.88 | 98 |

The concentrations of EF-Tu and [$^{3}$H]Phe-tRNA (1000 d.p.m./pmol) were 2.5 and 0.5 µM, respectively, in the RNaseA assay use to determine $k_1$. In the non-enzymatic-hydrolysis assay providing $T_1$, the concentrations were 1 and 0.25 µM, respectively. The $k_1$ value provided for free aa-tRNA corresponds to the maximum measurable dissociation rate constant. The limit is set by the rate at which the aa-tRNA is digested by RNaseA.

The protein concentrations, calculated from the resulting data, are those used in the subsequent characterizations.

The same assay was conducted in order to ensure that the times of incubation in further experiments were sufficient to reach equilibrium conditions. Samples were withdrawn at different times from 0 to 60 min.

Conversion of EF-Tu:GDP into EF-Tu:GTP

In order to convert the EF-Tu:GDP form into the EF-Tu:GTP form, 0.5 µM EF-Tu:GDP was incubated with 5 µM [$^{3}$H]GTP (6500–7500 d.p.m./pmol), 0.5 mM phosphoenolpyruvate and 0.062 µg/µl pyruvate kinase for 90 min at 0°C (the equilibrium
Fig. 3. Rate of GTP-hydrolysis, $v$, at 30°C in the presence and absence of kirromycin. Closed symbols in the presence of kirromycin: (●) wild type; (■) TuLys2Ala; (▲) TuLys2Glu; (●) TuArg7Ala; (▼) TuArg7Glu. The corresponding open symbols refer to the assay performed in the absence of kirromycin. All correlation coefficients are above 0.96. The EF-Tu concentration was 0.3 µM for all the experiments.

Fig. 4. Apparent dissociation rate constants for the ternary complexes at 0°C determined using the RNase A-protection assay. (●) wild type; (■) TuLys2Ala; (▲) TuLys2Glu; (●) TuArg7Ala; (▼) TuArg7Glu; (□) free aa-tRNA. $c_t$ and $c_0$ denote the concentration of ternary complex at time $t$ and the initial concentration, respectively. Correlation coefficients are all above 0.99. The results are given in Table V. The slope of the curve obtained for free aa-tRNA represents the maximum measureable dissociation rate constant. The restriction is due to the nature of the assay, which is limited by the rate of digestion of aa-tRNA by RNase A.

Fig. 5. Protection of Phe-tRNA$^{Phe}$ against non-enzymatic hydrolysis at 20°C: (●) wild type; (■) TuLys2Ala; (▲) TuLys2Glu; (●) TuArg7Ala; (▼) TuArg7Glu; (□) free aa-tRNA. $c_t$ and $c_0$ represent the concentration of ternary complex at time $t$ and the initial concentration of the complex, respectively. Correlation coefficients are all above 0.98. The results in terms of $T_2$ are given in Table V.

Fig. 6. Positions of Lys2 and Arg7 relative to aa-tRNA in the ternary complex, EF-Tu:GTP:Phe-tRNA$^{Phe}$. EF-Tu originates from *T. aquaticus*, while the Phe-tRNA$^{Phe}$ is from yeast.

Time obtained in an assay similar to that described above. The 50 µM GTP stock solution used in the assay was preincubated for 20 min at 20°C with 2.6 mM phosphoenolpyruvate and 0.1 µg/µl pyruvate kinase to avoid traces of GDP in the GTP.

Temperature stability

The temperature stability of the protein was measured in its complexes with GDP and GTP, the former by incubating 0.5 µM EF-Tu:GDP with 5 µM $[^{3}H]$GDP (1300 d.p.m./pmol) in binding buffer at different temperatures for 8 min. After this, the samples were kept on ice for 30 min and then filtered as described above.

The EF-Tu:GTP form first requires the conversion of all the bound GDP into GTP. Apart from this conversion, the assay was carried out as for the GDP form with the exception that the incubation on ice took place for 10 min.

**Dissociation rate constants for EF-Tu:GDP and EF-Tu:GTP**

In order to study the kinetics of dissociation, we incubated EF-Tu:$[^{3}H]$GDP or $[^{3}H]$GTP with unlabelled GDP or GTP in large excess. Under these conditions, the rebinding of $[^{3}H]$GDP or $[^{3}H]$GTP, once dissociated, is quenched by isotopic dilution (Arai et al., 1974).

The dissociation of the labelled nucleotide follows the equation of a first-order reaction, given by the expression
by the equation \( d \) can be neglected. If we integrate the equation it gives

\[
\ln\left(\frac{\gamma \cdot \text{GTP}}{\gamma \cdot \text{GDP}}\right) = k_\gamma \cdot t
\]

where \( k_\gamma \) is the dissociation rate constant for the ternary complex EF-Tu:GDP:yeast\[\text{Phe-tRNA}^{\text{Pr}}\] in E.coli. The dissociation rate constants were determined as described (Knudsen et al., 1995).

The dissociation rate constants were determined as described (Knudsen et al., 1995).

**Association rate constants for EF-Tu/GDP and EF-Tu/GTP**

The rate of formation of EF-Tu::GDP and GTP can be described by the equation \( dx/dt = k_{\gamma}(a - x)(b - x) - k_{\gamma}x \), where \( a = [1^\text{H}]\text{GDP}_0 \) is the initial concentration of radiolabelled GDP or GTP, \( b = [\text{EF-Tu}]_0 \) is the initial concentration of nucleotide-free EF-Tu and \( x = [\text{EF-Tu}:\text{GTP}] \), is the concentration of the complex with GDP or GTP at time \( t \) (Arai et al., 1974). However, for the initial phase of the reaction, the second term can be neglected. If we integrate the equation it gives

\[
\frac{1}{1}[\ln(a - x)/(b - x)] = k_{\gamma}t.
\]

For the determination of \( k_{\gamma} \) (GDP), 20 \( \mu \)l of nucleotide-free EF-Tu (~8–20 pmol depending on the mutant) was mixed with 980 \( \mu \)l of starting mixture containing \( [1^\text{H}]\text{GDP} \) (4000 d.p.m./pmol) to a final concentration of 0.04 \( \mu \)M GDP in GTPase buffer at an ice-cold 1:25 ml Eppendorf Combitip. Portions of 100 \( \mu \)l of the reaction mixture were spotted on nitrocellulose filters at different times from 0 to 200 s and washed as before. The radioactivity retained on the filters was then counted. The initial dissociation rate constant of nucleotide-free EF-Tu, \( k_{\gamma} \), was determined as described above.

For the determination of \( k_{\gamma} \) (GTP) was determined in the same way, but with the following exception: 150 \( \mu \)M \( [1^\text{H}]\text{GTP} \) (10 000 d.p.m./pmol) was incubated with 0.001 \( \mu \)g/\( \mu \)l pyruvate kinase and 700 nM phosphoenolpyruvate at 22°C for 15 min in a total volume of 985–990 \( \mu \)l in GTPase buffer and then put on ice for at least 10 min (starting mixture). Volumes of 10–15 \( \mu \)l of nucleotide-free EF-Tu (7–13 pmol) were mixed with the starting mixture and 100 \( \mu \)l portions were spotted, filtered, washed and counted as previously described.

**Preparation of nucleotide-free EF-Tu**

A 100 pmol amount of EF-Tu was mixed with 2% charcoal in 50 mM Tris–HCl, pH 7.6, at 4°C, 60 mM NH\(_4\)Cl, 10 mM EDTA, 1 mM DTT and 0.1% BSA in a total volume of 125 \( \mu \)l, kept on ice for 15 min and spun down at 13 000 r.p.m. at 4°C for 20 min. The supernatant was carefully withdrawn. The method was tested using radiolabelled EF-Tu:GDP to see if the GDP had been removed sufficiently and how much was still bound. At least 2% is necessary to retain protein stability whereas no more than 5% should be left in the preparation to be useful for the determination of the association rate constants, \( k_{\gamma} \) (GDP/GTP) (Fasano et al., 1982).

**GTPase activity**

The determination of the intrinsic GTPase activity is based on the measurement of the \( \gamma \cdot \text{GTP} \) liberated from \( [\gamma \cdot \text{GTP}] \) upon hydrolysis and extracted as a dodecamethylate complex in an acidic environment (Sanders et al., 1975).

EF-Tu:GDP and \( [\gamma \cdot \text{GTP}] \) were incubated separately with phospho-\( \text{Phe} \) and pyruvate kinase prior to the assay. All assays were carried out at 30°C. \( k_{\gamma} \) and \( k_m^\gamma \) were determined from Hanes plots, i.e. a plot of substrate concentration/reaction rate versus substrate concentration, \( V_{max} \) equals 1/slope and \( K_m/V_{max} \) equals the intercept with the ordinate.

The stimulation of the GTPase activity with 50 \( \mu \)M kirromycin was performed with 0.3 \( \mu \)M EF-Tu and a GTP concentration corresponding to the \( K_m^\gamma \) calculated for each protein. Kirromycin was added just before the two solutions were mixed. In this case, we simply compared velocities for similar reactions with and without kirromycin.

**The ternary complex**

The apparent dissociation rate constant, \( k_{\gamma} \), for the ternary complex EF-Tu:GTP:yeast\[\text{Phe-tRNA}^{\text{Pr}}\] was determined using the ribonuclease digestion rate assay (Louie and Jurnak, 1985) essentially as described by Andersen and Wiborg (1994) with the only exception that RNase A was added to a final concentration of 0.3 \( \mu \)g/ml.

The ability of the protein to protect the aminoacylated tRNA against non-enzymatic hydrolysis was measured as described (Pingoud et al., 1977; Andersen and Wiborg, 1994).

Prior to the assays, the \( \text{tRNA}^{\text{Pr}} \) was aminoacylated with \( [1^\text{H}]\text{Phe} \) as follows: 18 \( \mu \)M \( \text{tRNA}^{\text{Pr}} \) from yeast was incubated with 61 \( \mu \)M \( [1^\text{H}]\text{Phe} \) (935 d.p.m./pmol) and synthetase from yeast (obtained following the method of Von der Haar, 1979) in charging buffer (100 mM Tris–HCl, pH 7.6, at 4°C, 50 mM NH\(_4\)Cl, 12 mM MgCl\(_2\), 2 mM ATP, 1 mM DTT and 0.25 mM CTP) for 13 min at 37°C. Additional ATP up to 5 mM was also occasionally added. Prior to the non-enzymatic hydrolysis assay, the synthetase was removed by phenol extraction.

**Results**

**Purification, activity and stability**

The yield of protein varied among the mutants and was in the range 0.3–1.3 mg protein per gram wet cell paste. The purity of the protein, determined by SDS–PAGE, was always about 95%. The protein activity varied from 42 to 72% as calculated from the GDP binding assay.

In the temperature stability assay, the ability of the proteins
to maintain their structure and function when the temperature was increased continuously was examined. We calculated $\phi$, which is the temperature at which the activity of the protein is 50% of the maximum under the given conditions. The results showed no significant differences between the mutants and the wild type (data not shown).

**Interactions with GDP and GTP**

The nucleotide association and dissociation rate constants were determined as described in Materials and methods and the results are presented in Tables I and II and Figures 1 and 2.

The dissociation rate constants of all the mutants are almost the same for both GDP and GTP when compared with the wild type. Nevertheless, TuArg7Ala and TuArg7Glu present a very slight tendency for a lowered binding of GTP, which is reflected in the increased $k_{-1}(GTP)$ values. In contrast, $k_{-1}(GDP)$ and $k_{-1}(GTP)$ for TuArg7Glu are ~30 and 10 times lower than those of the wild type, while for the other mutants the values resemble those of the wild type. TuArg7Ala and TuLys2Glu show some subtle differences although not as dramatic as those mentioned for TuArg7Glu. We have observed that when we make these two mutants nucleotide free, they become unstable very quickly. This is not surprising when we consider that the wild type form is stable for only 2 h under the same conditions.

In summary, the introduction of a glutamic acid instead of an arginine at position 7 produces a pronounced effect on nucleotide binding. In comparison, the effect is mild on introducing an alanine.

**GTPase activity**

The capacity of the proteins to sustain GTP hydrolysis was analysed in both the presence and absence of kirromycin. Table III summarizes the results from the experiment carried out to obtain $k_{cat}$ and $K_m$ values. The results in Table III show no significant differences between the wild type and the mutants. TuLys2Glu shows a slight increase in both $k_{cat}$ and $K_m$, but $k_{cat}/K_m$ is very similar to that of the rest of the mutants. The fact that the GTPase assay was carried out under slightly different conditions for this mutant (0.5 $\mu$M EF-Tu instead of 0.3 $\mu$M, which was used for the wild type and the other mutants) might account for the discrepancy.

Figure 3 shows the initial rate, $v$, for wild type and the mutants in the presence and absence of kirromycin. The GTP concentrations employed are those of the individual $K_m$ for each protein. Table IV summarizes the results. The relative effects for all the mutants are almost the same as that of the wild type. The fact that stimulation is observed also suggests that kirromycin binds to the mutants, indicating that the overall structure is conserved for the mutant proteins.

**The ternary complex**

The formation of the ternary complex, EF-Tu:GTP:aa-tRNA, was characterized in two different ways. The apparent dissociation rate constants, $k_{-1}$, found using the RNaseA digestion assay and $T_1$, representing the time at which aa-tRNA has undergone a 50% spontaneous deacylation, are calculated from the plots shown in Figures 4 and 5, respectively.

From the results shown in Table V and directly from the Figures 4 and 5, it can be clearly seen that the ternary complexes, EF-Tu:GTP:aa-tRNA, formed with the mutants TuArg7Ala and TuArg7Glu possess almost the same properties as the free aa-tRNA under the conditions applied in the assay. This means that the Arg7 mutants offer no protection to aa-tRNA, i.e. the binding of aa-tRNA, if any, is very weak. In the case of TuLys2Ala and TuLys2Glu there is a 2.5-fold decrease in $k_{-1}$ compared with the wild type and a slight decrease in the $T_1$ values. It is important to point out that both assays, although based on different principles, show the same results.

**Discussion**

The GDP exchange assay and the equilibrium test showed that GDP and GTP can bind to the mutants. The temperature stability assays for GDP and GTP did not reflect any major differences between wild-type EF-Tu and the mutants. We therefore conclude that the overall structure is conserved for the mutant proteins.

The results from the ternary complex assays (ribonuclease digestion and non-enzymatic hydrolysis assay) show that Lys2 is not essential for the binding of aa-tRNA and that the two mutants of Arg7 cannot bind tRNA. These results from TuArg7Ala and TuArg7Glu can have two possible explanations: either the interaction between aa-tRNA and EF-Tu is disturbed directly by the mutation, or a conformational change makes it impossible for EF-Tu:GTP to bind aa-tRNA, i.e. the effect is indirect. In both cases the changes must be localized, i.e. the overall structure is maintained.

The structure of the ternary complex Phe-tRNA$^{Phe}$-EF-Tu:GTP (Nissen et al., 1995) was solved recently in our department. In contrast to the earlier structures of EF-Tu:GDP and EF-Tu:GTP (Kjeldgaard and Nyborg, 1992; Berchtold et al., 1993; Kjeldgaard et al., 1993), the new structure clearly shows the positions of the side chains of interest relative to the Phe-tRNA$^{Phe}$. The side chain of Lys2 points away from the protein and out into the solvent and there is no possible direct contact with Phe-tRNA$^{Phe}$ (Figure 6) or with any residues within the protein. In the case of TuLys2Ala and TuLys2Glu, the slight increase in $k_{-1}$ and $T_1$ are likely to be produced because the substitution of amino acids always generate some very slight distortions in the angles of the backbone, especially in a rather flexible structure such as that at the N-terminal region. Some rearrangement of the water molecules around the side chain is also possible, but this phenomenon cannot account for the effect detected.

Figure 7 shows the most interesting finding. In the Thermus aquaticus EF-Tu:GTP form found in the ternary complex, the nitrogens of the guanidine groups of Arg7 are 2.44 and 3.21 Å apart, respectively, from the oxygens of the carboxyl group of Asp284 (Glu272). These are suitable distances for the formation of a double salt bridge between the two side chains. The numbers in parentheses correspond to the residues in E.coli. Asn285 (Asn273) immediately following Asp284 (Glu272), is part of the pocket that accommodates the 20 possible amino acids of aa-tRNA when bound to EF-Tu:GTP. Leu277 (Leu265) and Ile281 (Arg269) provide a hydrophobic environment and shield the salt bridge from the solvent. The presence of these salt bridges maintain domains 1 and 2 tightly together.

Moreover, the results from Lautberg (Lautberg et al., manuscript in preparation) showing that Lys9 binds to domain 1 in the GTP form via a salt bridge to Glu71 (Glu70) indicate that the N-terminal fragment 7–9 needs to be bound tightly to both domains 1 and 2 at the same time and in a very specific way in order to make ternary complex formation possible, as the absence of any of these two bonds significantly impairs aa-tRNA binding. This suggestion is supported by the fact that Asp284 (Glu272) is conserved as an acidic residue (Asp or
Glu) among the different species of EF-Tu and EF-1a. It is important to realize that in the GDP form, residues Arg7 and Asp284 (Glu272) have no contact with each other, as the distance between them is at least 10 Å.

Figure 7 shows residues 2 and 7 in the ternary complex. They are both at the opposite site of the aa-tRNA binding site of EF-Tu. Others have also shown that residues not in direct contact with aa-tRNA can have an influence on its binding as exemplified in the following. Several types of studies have pointed out His118 as being involved in the binding of aa-tRNA (Jonak et al., 1984, 1994; Metz-Boutigue et al., 1989; Andersen and Wiborg, 1994). However, this residue is buried in the domain I/III interface and completely inaccessible to aa-tRNA, which is at least 16 Å away (Nissen et al., 1995).

Other mutants, which were selected as being resistant to kirromycin, were also found to be defective in their interaction with aa-tRNA. The most strongly affected mutants were TuGln124Arg and Leu120Gln, which are both located at the interface between domains I and III (Abdulkarim et al., 1996). Similarly, phosphorylation of EF-Tu, which takes place at the interface between domains I and III, also prevents ternary complex formation (Alexander et al., 1995).

The other results that need some explanation are those concerning association and dissociation constants for GDP and GTP. The results for TuLys2Ala and TuLys2Glu are very similar to those obtained for the wild type, except that the $k_{-1}$ (GTP) for TuLys2Glu differs slightly. However, for TuArg7Ala and especially for TuArg7Glu the results show some significant differences.

The low value of $k_{-1}$ (GDP) for TuArg7Glu is not caused by the absence of binding, but is due to a very slow process compared with those rates obtained for the wild type and the other mutants. In the GTP forms of TuLys2Glu, TuArg7Ala and TuArg7Glu, however, it seems as if the nucleotide-free mutant species are impaired in their ability to revert to the stable conformation. There is some binding, but it seems that the main part of the population of the nucleotide-free molecules cannot attain the precise structure necessary to bind GTP. The introduction of a charged side chain (Glu) provides the possibility of making interactions with the side chains close to it in the disordered, nucleotide-free structure. These new interactions together with those of the unpaired residues, for example that of Asp284 (Glu272), could account for this extra degree of instability and are enough to impair the association with GTP and retard GDP binding.

In agreement with this, we tested whether the addition of EF-Ts could improve the GTP binding in an experiment similar to that used for measuring temperature stability. EF-Ts binds to TuArg7Ala and TuArg7Glu in the GDP form and enhances the exchange of GTP for GDP (data not shown). However, when the experiment was performed with the nucleotide-free form of these mutants there was no enhancement at all. We believe that the structural changes caused by the mutation of Arg7 become much more pronounced in the nucleotide-free form and reach a point where the binding of EF-Ts is so strongly impaired that no binding takes place at all. As a consequence, no enhancement of nucleotide binding is observed. If the reason for the lack of enhancement of nucleotide binding was the formation of a more stable EF-Tu:EF-Ts complex, this would most likely also have been observed when using EF-Tu:GDP as the starting point. At the moment, we are unable to explain the effect in structural terms.

The effects on both the ternary complex formation and the association with GTP lead us to propose an aspect of the Arg7–Asp284 (Glu272) salt bridge related to the molecular switch of EF-Tu. Prior to the switching from the GDP form to the GTP form, domains 1 and 2 are far apart. The conformational changes, initiated in helix B of domain 1, propagate to the rest of the molecule and two of the last rearrangements are probably those of Arg7 and Lys9. If these salt bridges were essential for the molecule, then GTP binding, kirromycin binding, EF-Ts binding and the GTPase activity of the mutants would have been impaired completely. This is not the case, however, and we therefore propose that the fixation of domain 2 to domain 1 through the N-terminal region is the last step in the rearrangement of the GDP form to the GTP form. In between, a succession of different stages drive the molecule to the final compact GTP form that we know from the available crystallographic structures (Kjeldgaard and Nyborg, 1992; Berchtold et al., 1993, Kjeldgaard et al., 1993).

In summary, the following conclusions have been drawn on the basis of the results presented. When the interaction between Arg7 and Asp284 (Glu272) is disrupted, the capacity for binding aa-tRNA is almost lost. Lys2 is not directly involved in aa-tRNA binding. Arg7 works as a staple between domains 1 and 2 in the GTP form of EF-Tu maintaining residues 7–9 in a very specific position. The nucleotide-free forms of the mutants TuArg7Ala, TuArg7Glu and TuLys2Glu are very unstable. The impairment of $k_{-1}$ (GTP) for the Arg7 mutants although with the conservation of their overall structure, their nucleotide and kirromycin binding capacity and GTPase activity raise the possibility of a sequential propagation of the molecular switch from the GDP form to the GTP form where Arg7 participates in one of the last steps.

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