Mapping Escherichia coli Elongation Factor Tu Residues Involved in Binding of Aminoacyl-tRNA*

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Ove Wiborg‡§, Carsten Andersen‡, Charlotte R. Knudsen‡, Brian F. C. Clark‡, and Jens Nyborg‡

From the ‡Department of Chemistry, Aarhus University, Langelandsgade 140, 8000 Aarhus C and §Cytogenetic Laboratory, Psychiatric Hospital, Skovagervej 2, 8240 Risskov, Denmark

Elongation factor Tu (EF-Tu) is a key enzyme in protein biosynthesis. In its active conformation with GTP as a cofactor, it carries aminoacyl-tRNA (aa-tRNA) to the A-site of the programmed ribosome in the process of decoding mRNA (1). The complex of EF-Tu, GTP, and aa-tRNA has been denoted the ternary complex. During peptide bond formation with GDP as a cofactor, the ribosome and aa-tRNA act as stimulators of the GTPase activity at this crucial step. In Escherichia coli, peptide bond formation proceeds at a rate of 10–20 bonds/s/ribosome (2).

E. coli EF-Tu is a monomeric protein consisting of 393 amino acid residues and with a molecular mass of 43.2 kDa. The nucleotide sequences of the two EF-Tu encoding genes tufA and tufB have been determined (3, 4), and so has the amino acid sequence of EF-Tu (5). The two genes differ only in 13 nucleotide positions, and the two gene products are identical except for the C-terminal position, which is a glycine in the tufA gene product and a serine in the tufB product. The existence of two almost identical genes encoding EF-Tu has been suggested to be part of a complicated bioregulatory mechanism (6).

EF-Tu is a member of the guanine nucleotide-binding proteins (G-proteins), which include the Ras superfamily and the heterotrimeric G-proteins (7). The G-proteins are all GTPases and consequently the capability to cycle between a GTP-bound “on-state” and a GDP-bound “off-state.” The G-domain has thus been described as a molecular switch (8, 9).

The crystallographic structure of EF-Tu has been solved in complex with both GDP and a GTP analogue (10–12). The structures of the related G-proteins p21 Ras, and transducin (14, 15), in complex with both GDP and a GTP analogue, are also known. The structure of the G-protein elongation factor G has been solved in a nucleotide-free form and in complex with GDP (16, 17). The structure of the a subunit of the heterotrimeric G-protein, Gt12, has been solved in complex with GTP and a transition state analogue (18).

For representatives of the three main groups of G-proteins, where both the active and inactive conformations are known, EF-Tu, p21 Ras, and transducin, there seems to be a distinct mechanism for the conformational change from the GTP to the GDP conformation. Nevertheless, for all these proteins one of the major structural changes involves the helix following the second nucleotide interacting loop. In EF-Tu this helix is named helix B.

Helix B is flanked by two strictly conserved glycines in the Ras-like proteins and EF-Tu. In EF-Tu an important structural role in the switch mechanism has been inferred for the glycines (19, 20). In the GDP-form of E. coli EF-Tu, helix B is flanked by Gly-83 and Gly-94. The conformational change involving helix B resembles the changes in p21 Ras. During activation an additional helical turn is formed at the carboxyl end of helix B at the expense of a turn at the beginning of the helix. Furthermore, the direction of helix B is dynamically shifted by an angle of 42° (11). The shift in conformation also involves the movement of domain 1 relative to domains 2 and 3 corresponding to a rotation of 90.8° in relative orientation (11). As a result the big cleft between domain 1 and 2 is diminished; new side chains become solvent-exposed, and the overall shape turns more globular in the GTP form.

In the GTP form helix B comprises residues 87–97 (YVKKN-MTGAAQ). The helix B region is evolutionarily well conserved for prokaryotic EF-Tu as well as its eukaryotic counterpart EF-1α. Lys-89 and Asn-90 are two of the most conserved residues, and in the GTP form their side chains are solvent-exposed with good inherent potentials for formation of secondary bonds to ligands.
Elongation Factor Tu Mutants with Decreased tRNA Affinity

To elucidate their functional role in tRNA binding, Lys-89 and Asn-90 have been subjected to mutational analysis. Each of these residues has been cross-linked to aa-tRNA, indicating that His-66 is in close proximity to the aminoacyl moiety of aa-tRNA (26). The cross-linking sites potentially only about 7 Å long, and the cross-linking sites potentially for formation of the ternary complex EF-Tu GTP (0.05–2 μM) were identified by single-stranded DNA sequencing of the M13mp11TuA constructs, using Sanger's deoxy chain-termination method (32) and confirmed on the expression vector, pGEFXTuA, by the double-stranded DNA cycle sequencing method (33).

Expression and Purification—Expression and purification of mutant proteins was done as described earlier (29).

Protein concentrations were determined by a modification of Bradford (34) using bovine serum albumin as a standard. Alternatively, EF-Tu concentrations in high purity solutions were calculated from the absorption at λmax using a specific extinction coefficient of 4 × 10^4 M^-1 cm^-1 (35). The yields of the purified mutant proteins were in the range of 1 mg/g wet cell paste.

Protein Activity and Stability—GDP binding activity was taken as an overall measure of EF-Tu activity, although the ability of EF-Tu to bind nucleotide is retained longer than the ability to bind aa-tRNA, as can be seen from the characterization of the ternary complex. GDP binding activity was measured upon equilibration with [3H]GDP (specific activity 700 cpm/pmol) and binding to nitrocellulose filters, essentially performed as described by Miller and Weissbach (35). The thermal inactivation profiles for the GDP and GTP form were determined as described by Gümsel et al. (36). 10 pmol of EF-Tu were incubated with 200 pmol of [3H]GDP/GTP in 50 μl of binding buffer (50 mM Tris/Cl, pH 7.6, 100 mM NH4Cl, 10 mM MgCl2, 50 mM KC1, 1 mM DTT). The mixture was incubated for 8 min at the assay temperature and then placed on ice for 30 min. Chemical stability toward denaturation by urea was determined as described by Harnack et al. (37). 0.3 pmol of EF-Tu were preincubated with either 15 μM [3H]GDP or a mixture containing 367 μM theophylline, 13 μM pyruvate kinase, and 15 μM [3H]GTP for 20 min at 20°C. 12 pmol of EF-Tu-GDP/GTP were incubated with increasing amounts of urea (0–3 M) in a total volume of 60 μl for 45 min at 20°C, and then placed on ice before filtration.

GTPase Activity Assay—The GTPase activity was measured as the amount of liberated inorganic phosphate, using the isopropyl acetate/molybdate method (38).[3H]GTP and EF-Tu-GDP were preincubated separately with phosphoenolpyruvate and pyruvate kinase prior to the assay. 45 pmol of EF-Tu-GTP were incubated at 20°C in 50 mM Tris/Cl, pH 7.6, 5 mM MgCl2, 50 mM KC1, 0.5 mM DTT, and 1.5–30 μM of [γ-32P]GTP (specific activity 1700 cpm/pmol). Samples were withdrawn every 5 min up to 30 min and the reaction stopped by addition of perchloric acid. Kcat and K∗ were determined from Lineweaver-Burk plots. For further characterization of the GTPase activity, 0.5 μM EF-Tu was incubated with 60 μM GTP and stimulated by addition of either 0.1 μM EF-Ts, 50 μM kirromycin, 0.15 μM aa-tRNA, or 0.15 μM poly(U)-programmed ribosomes. Programmed ribosomes were blocked in the P-site with uncharged tRNA^Phe. Reactions were stopped with perchloric acid and ribosomes were precipitated by a quick centrifugation step prior to extraction of liberated inorganic phosphate.

Characterization of the Ternary Complex—In the following assays EF-Tu-GDP was preincubated with GTP, phosphoenolpyruvate, and pyruvate kinase. Yeast tRNA^Phe was aminated by incubating 14 μM tRNA^Phe with 50 μM [3H]Pho, 5 mM ATP, 0.24 mM CTP, and 1 μM of synthetase in 250 μl of changing buffer (100 mM Tris/Cl, pH 7.5, 50 mM NH4Cl, 12 mM MgCl2, 2.8 mM β-mercaptoethanol) at 37°C for 10–15 min. The charging efficiency of the Phe-tRNA^Phe used was above 80%. The synthetase was purified crudely from Saccharomyces cerevisiae according to von der Haar (39). For use in the nonenzymatic hydrolysis assay, the synthetase was removed after charging by phenol extraction, and Phe-tRNA^Phe was precipitated.

The equilibrium dissociation constant Kd for formation of the ternary complex EF-Tu-GTP [3H]Phe-tRNA^Phe was determined by the ribonucleic acid assay essentially as described by Louie and Jurnak (40). The concentrations of EF-Tu and aa-tRNA were, however, increased to the μM range due to a considerably higher Kd for the mutants. 0.67 μM [3H]tRNA^Phe (specific activity 374 cpm/pmol) was incubated with EF-Tu-GTP (0.05–2 μM) in 50 μl of PH-6 buffer (60 mM Tris/Cl, pH 7.8, 30 mM KCl, 30 mM NH4Cl, 6 mM MgCl2) at 0°C for 30 min. 1 μl of RNase A (10 mg/ml) was added for 15 s and the amount of ternary complex measured after precipitation, filter binding, and washing. The Kd values were determined from Scatchard plots (41).

The ternary complex dissociation rate constant κ− was determined

EXPERIMENTAL PROCEDURES

Construction of Mutants—Site-directed mutagenesis was performed according to Taylor et al. (28). An M13mp11 done containing the tuF

gene was used as a template (29). The sequence of the mutagenic primer for mutating Lys-89 was 5'-GACTATGTGGMGAACATGAC-3', and for mutational analysis of Asn-90 5'-GACTATGTAAAAGMGAATGCAC-3', where M is C or A for mutating to alanine or glutamate, respectively. Upon mutagenesis the tuA mutant gene was recloned and expressed in the pGEK gene fusion system (30). The recA strain JM109 (31) was used as a host.

Expected mutations were identified by single-stranded DNA sequencing of the M13mp11TuA constructs, using Sanger's deoxy chain-termination method (32) and confirmed on the expression vector, pGEFXTuA, by the double-stranded DNA cycle sequencing method (33).

FIG. 1. Structural cartoon of EF-Tu from Thermus aquaticus in complex with GTP. Using E. coli EF-Tu numbering locations of His-66, Lys-89, Asn-90, and His-118 side chains are shown as are the positions of the nucleotide and Mg^2+ (black ball). Helix B is black, and the domain numbering is indicated.

To map the interaction points of EF-Tu and aa-tRNA, different chemical modification studies have been applied in addition to cross-linking and site-directed mutagenesis studies.

Mutation studies on the ternary complex have revealed that His-66 and His-118 and at least one of the methionines are protected against chemical modification (23, 24). No unequivocal determination of macromolecular recognition interaction sites has so far been established.

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In another attempt EF-Tu fragments 56–68 and 118–124 were identified as cross-linking to aa-tRNA (26). The cross-linking agent was trans-diaminedichloroplutonium (II), which is only about 7 Å long, and the cross-linking sites potentially His-66 and His-118, respectively.

Enzymatic characterization of EF-Tu mutants in which His-66 was mutated to Ala and His-118 replaced by either Ala or Glu have confirmed the results from chemical studies (27).

In this report we have extended the characterization of these mutants to include studies on their ability to sustain poly(Phe) synthesis.

The functional role of Lys-89, Asn-90, His-66, and His-118 is discussed in relation to structural analysis of the ternary complex.

Experiment 1

Construction of Mutants—Site-directed mutagenesis was performed according to Taylor et al. (28). An M13mp11 clone containing the tuF
by the ribonuclease-digestion rate assay as described by Louie and Jurnak (40). In 100 μl of PP-6 buffer 2.5 μM EF-Tu-GTP was incubated with 0.7 μM [3H]Phe-tRNA<sub>Phm</sub> (specific activity 574 cpm/μmol) at 0°C for 30 min. At time 0 RNase A was added to a final concentration of 30 μg/ml. Aliquots of 15 μl were successively withdrawn, precipitated, and filtrated. As the dissociation of the complex follows first order kinetics, k<sub>t</sub> may be determined as the slope of a plot of ln(EF-Tu-GTP-Phe-tRNA<sub>Phm</sub>/M) versus time.

The protective effect of EF-Tu in the ternary complex against non-enzymatic hydrolysis of the aminoacyl bond was measured as previously described by Piggins and Urbanke (42). In 200 μl of PP-6 buffer 1 μM EF-Tu-GTP is incubated with 0.3 μM [3H]Phe-tRNA<sub>Phm</sub> (specific activity 574 cpm/μmol) at 0°C for 30 min. Following incubation at 20°C aliquots of 20 μl were withdrawn, precipitated, and filtrated. From a plot of c<sub>c</sub>/c<sub>0</sub> versus time, where c<sub>c</sub> and c<sub>0</sub> denote the concentrations of the ternary complex at time t and 0, respectively, the negative slope is taken as a measure of the protective effect of EF-Tu on the aminoacyl bond.

In Vitro Synthesis of Poly(Phe)—The poly(Phe) assay was performed in the polynuc-frame as described by Ehrenberg et al. (43). The assay conditions were optimized to ensure a linear relationship between the rate of poly(Phe) synthesis and the amount of EF-Tu. The composition of ribosome mix (in polymix buffer) was 2.6 mg/ml poly(U), 3.75 μM ribosomes, and 1.7 μM [14C]Phe-tRNA<sub>Phm</sub> (specific activity 31 Ci/mmol). The factor mix (in polybuffer) contained 2 mM ATP, 20 mM phosphoenolpyruvate, 100 μM GTP, 0.1 mg/ml pyruvate kinase, 0.006 mg/ml myokinase, 14.6 μM [14C]Phe-tRNA<sub>Phm</sub> (specific activity 31 Ci/mmol), 0.2 mg/ml EF-Ts, 0.1 mg/ml EF-G, and 30 nM EF-Tu. The tRNA was charged before the mixture were incubated separately at 30°C for 15 min. At time 0, equal volumes of the preincubated mixtures were combined. Samples were withdrawn at appropriate time intervals up to 15 min and precipitated in 10% trichloroacetic acid. The samples were placed at 85°C for 30 min followed by 5 min on an ice and finally filtered.

EF-G was purified as described by Bodley et al. (44) and Leder (45), whereas EF-Ts was a gift from the G. E. Brown. Ribosomes were purified from E. coli MRE600 cells grown to an OD<sub>260</sub> of 2–2.5 in a medium containing 0.4–0.5 M NH<sub>4</sub>Cl, and centrifuged (40,000 × g, 16 h). The ribosomal pellet was resuspended in buffer 2 (10 mM Tris-Cl, pH 7.6, 4°C, 10 mM MgCl<sub>2</sub>, 30 mM NH<sub>4</sub>Cl, 1 mM DTT) and opened in a French press. The cell debris was removed by centrifugation (18,000 × g, 30 min). The ribosomes were pelleted by ultracentrifugation of the supernatant (50,000 × g, 2 h). The ribosomal pellet was carefully resuspended in buffer 1, layered onto a 30% sucrose cushion in buffer 1 containing 0.4–0.5 M NH<sub>4</sub>Cl, and centrifuged (40,000 × g, 16 h). The ribosomal pellet was resuspended in buffer 2 (10 mM Tris-Cl, pH 7.6, 4°C, 10 mM MgCl<sub>2</sub>, 30 mM NH<sub>4</sub>Cl, 1 mM DTT, 10% glycerol), frozen in liquid nitrogen, and stored at −80°C (47). The concentration was calculated from the absorbance at A<sub>260</sub> (1 A<sub>260</sub> = 23 pmol).

RESULTS

Protein Purification, Activity, and Stability—To prepare EF-Tu and mutants thereof in large amounts of high purity and high enzymatic activity, the proteins were expressed as recombinants in the prokaryotic pGEX-gene fusion system (30). This system allows a one-step affinity purification of recombinants and subsequent release of EF-Tu upon cleavage of fusion protein with blood clotting factor X<sub>a</sub> (29). We have shown that recombinant wild type EF-Tu possesses the same enzymatic characteristics as authentic EF-Tu according to thorough in vitro characterization (data not shown). The final yield of purified recombinant EF-Tu was above 1 mg/g wet cell paste. The purity of protein preparations was above 98% as determined by SDS-polyacrylamide gel electrophoresis. The enzymatic activity as measured by nucleotide binding capability was above 90%. Wherever given protein concentrations are in units of EF-Tu active in nucleotide binding.

The half-lives of irreversible chemical and thermal denaturation were determined for both the EF-Tu-GDP and EF-Tu-GTP forms (Table I). The results were used as indicative measures of structural stability and as guidelines for selection of optimal assay temperatures. As a consequence all experiments were carried out at a maximum of 30°C, which is at a temperature with full enzymatic activity for all mutants.

GTPase Activity—The kinetic constants k<sub>m</sub>, and k<sub>cat</sub>, for the intrinsic GTPase activity were determined from Lineweaver-Burk plots. The plots were constructed from initial rates, i.e. less than 1 mol of GTP/mol of EF-Tu was hydrolyzed. Plots of initial rates at different substrate concentrations all had correlation coefficients above 0.98.

k<sub>m</sub> and k<sub>cat</sub> values summarized in Table II show only minor variations compared with wild type. The ratio k<sub>cat</sub>/k<sub>m</sub>, which incorporates substrate affinity and catalytic capability of the enzyme into a single factor, indicates that N90E possesses lower intrinsic GTPase activity.

Stimulation of GTPase activity is an important event that occurs in order to speed up the rate of translation in vivo. We examined this mechanism with a number of stimulators, both separately and in different combinations. The effects measured at a fixed substrate concentration appear to be almost identical for mutants and for wild type (data not shown).

In Vitro Translation Assay—The ability of the mutants to sustain poly(U)-directed poly(Phe) synthesis was tested both in the presence and absence of nucleotide exchange factor Ts (Table III). The incorporation rates for Asn-90 mutants are the same as for wild type. This also holds for the Lys-89 mutants when EF-Ts is included. Surprisingly, however, when EF-Ts is omitted the rate is about twice as high for Lys-89 mutants as for wild type.

The mutants H66A, H118A, and H118E have been thoroughly characterized as reported in a previous paper (27). Here additional data are presented on their activity in the in vitro translation assay (Table III).

For H66A the incorporation rates are as for wild type. However, for the His-118 mutants the rates upon EF-Ts stimulation are considerably lower.

In characterization of the affinity for tRNA the apparent dissociation rate constant, k<sub><sub>2</sub></sub>, was determined using the ribonuclease-digestion rate assay (40). A decisive step in this assay is adjustment of the RNase level. RNase is omitted in order to prevent reassociation taking place but can in excess potentially also degrade tRNA in complex with EF-Tu. k<sub><sub>2</sub></sub> was calculated from plots of ln([EF-Tu-GTP-Phe-tRNA<sub>Phm</sub>]/M) versus time (Table IV).

In a different strategy RNase was omitted. This allows some reassociation to occur while measuring the rate of nonenzymatic hydrolysis of the aminoacyl bond. Rates of nonenzymatic hydrolysis were calculated from plots of c<sub>c</sub>/c<sub>0</sub> versus time, where c<sub>c</sub> and c<sub>0</sub> denote the concentrations of the ternary complex at time t and 0, respectively (Table IV).

The relative ratios of dissociation rates for ternary complexes, when comparing wild type and mutants, are approximately the same using either of the two different assay methods. The results taken together indicate a reduction in tRNA affinity upon mutation of either Lys-89 or Asn-90, in particular when mutating to glutamate.

The equilibrium dissociation constant, K<sub>d</sub>, for the ternary

| EF-Tu type | k<sub>2</sub> GDP | k<sub>2</sub> GTP | γ<sub>2</sub> GDP | γ<sub>2</sub> GTP |
|------------|----------------|--------------|--------------|--------------|
| wt EF-Tu   | 51             | 45           | 2.8          | 1.2          |
| K89A       | 45             | 45           | 1.7          | 1.2          |
| K98E       | 45             | 45           | 1.7          | 1.0          |
| N90A       | 58             | 45           | ND           | ND           |
| N90E       | 50             | 40           | ND           | ND           |

Table I: Thermal and chemical stability
complex was determined from Scatchard plots for wild type and K89A (Fig. 2 and Table IV). For K89E, N90A, and N90E the values were, however, below the detection limit for this method. This also indicates a reduced tRNA affinity and is in accordance with considerably higher kcat values for the corresponding mutant-tRNA complexes. Using the ribonuclease assay the highest value measurable in our hands was 300 nM.

**DISCUSSION**

Thermal and chemical stability assays indicate K89A and K89E to be slightly more unstable in the GDP form compared with wild type (Table I). Structural determination of EF-Tu GDP indicates the presence of a salt bridge between Lys-89 and Asp-315 (10). This salt bridge is, however, not present in the GTP form (11). The concern for the K89E mutant is that a lysine residue was substituted by a glutamate, thus replacing the electrostatic attraction with repulsion. Also for K89A the possibility of salt bridge formation with Asp-315 was disrupted due to the substitution.

From Table II the intrinsic GTPase activity given as kcat/Km appears to be maintained for K89A, K89E, and N90A, i.e. neither nucleotide affinity nor the hydrolytic mechanism is affected by the mutations. Only for N90E a slight decrease is observed, which is primarily due to a lower GTP affinity as reflected by an increase in Km. A lower GTP affinity of N90E could also explain a reduced thermal stability of the GTP form of this mutant (Table I). Stimulation of GTPase activity seems essentially maintained for all mutants (data not shown). It should be noted, however, that the strong stimulatory effect of kirromycin is less pronounced for Lys-89 mutants.

The biological function of EF-Tu, as measured by its ability to sustain peptide synthesis, was tested in an in vitro translation assay. The mutants H66A, H118A, and H118E, which all have been described in a previous paper (27), were also tested in this assay (Table III). When nucleotide exchange factor EF-Ts was included in the assay all mutants, except the two His-118 mutants, sustained poly(Phe) synthesis at approximately the same rate as wild type. In contrast to this, His-118 mutants show drastically reduced activities. Also without EF-Ts the poly(Phe) synthesis rate is significantly reduced for K89A (Ef-Tu-Ts, when comparing to GDP binding (set to 100%).

**Table II**

| EF-Tu type | Km (µM) | kcat × 10⁹ | kcat × 10⁹/Km |
|------------|--------|-------------|---------------|
| wt EF-Tu   | 9.8 ± 0.59 | 182 ± 36.3 | 19           |
| K89A       | 16.3 ± 0.94 | 194 ± 38.1 | 12           |
| K89E       | 8.3 ± 0.76  | 126 ± 28.2  | 15           |
| N90A       | 5.3 ± 0.86  | 70 ± 9.43   | 13           |
| N90E       | 33.7 ± 13.7 | 162 ± 26.6  | 5            |

**Table III**

| Incorporation rates of Phe, in the presence and absence of EF-Ts, are calculated. Standard deviations are given. wt, wild type. |
|----------------------------------------------------------|
| EF-Tu type | Incorporated Phe/EF-Tu (mol/mol/min) |
| Without EF-Ts | With EF-Ts |
|----------------|-------------------|
| wt EF-Tu       | 0.9 ± 0.19        | 16 ± 3.1 |
| K89A           | 2.6 ± 0.25        | 19 ± 0.1 |
| K89E           | 2.3 ± 0.24        | 13 ± 1.0 |
| N90A           | 0.2 ± 0.04        | 10 ± 0.7 |
| N90E           | 1.2 ± 0.15        | 14 ± 1.7 |
| H66A           | 1.0 ± 0.20        | 17 ± 2.7 |
| H118A          | 1.0 ± 0.01        | 7 ± 0.35 |
| H118E          | 0.3 ± 0.41        | 2 ± 0.05 |

**Table IV**

| Kinetic constants for the ternary complex |
|------------------------------------------|
| EF-Tu type | kcat × 10⁵ | Km | Nonenzymatic hydrolysis rate |
|------------|------------|----|-----------------------------|
| wt EF-Tu   | 0.3 ± 0.08 | 12 ± 0.02 | 0.11 ± 0.02 |
| K89A       | 1.3 ± 0.28 | 94 ± 0.04 | 0.21 ± 0.01 |
| K89E       | 7.2 ± 0.33 | >300 | 0.43 ± 0.02 |
| N90A       | 3.5 ± 0.08 | >300 | 0.25 ± 0.03 |
| N90E       | 4.5 ± 0.05 | >300 | 0.35 ± 0.04 |

**FIG. 2.** Scatchard plot for determination of Km for the ternary complex. A and B are for wild type EF-Tu and [K89A]EF-Tu, respectively. v denotes the average number of Phe-tRNA/Phe molecules bound per molecule EF-Tu. The maximal value of v is given by the abscissa intercept. A value below 1 indicates that the protein preparation is not fully active in tRNA binding. 70% for wild type EF-Tu and 35% for [K89A]EF-Tu, when comparing to GDP binding (set to 100%).
when comparing to wild type (Table I). Thus a relatively higher stability of the GTP form could explain a higher unstimulated nucleotide exchange rate for the Lys-89 mutants. Helix B, which includes Lys-89, could on the basis of these results be suggested to be part of a target region of nucleotide exchange factor EF-Ts.

The tRNA affinity was significantly reduced for all mutants as measured in three different assays. The equilibrium dissociation constant, \( K_d \), the dissociation rate constant, \( k_{-1} \), and the nonenzymatic hydrolysis rate all show a reduction in tRNA affinity upon mutation of either Lys-89 or Asn-90 (Table IV).

The importance of Lys-89 and Asn-90 is emphasized by their location within helix B and by their evolutionary conserved nature.

During the structural switch to the GTP form, i.e. the one active in tRNA binding, helix B undergoes some remarkable overall changes. With respect to amino acid numbering helix B is moved by one turn of a helix toward the C terminus, and more dramatically, the direction of the helix is shifted by an angle of \( 42^\circ \) (11).

It should be noted that during the structural switch the side chains of Lys-89 and Asn-90 in the GTP form becomes solvent-exposed, thus the \( \epsilon \)-amino group of Lys-89 seems to interact with water molecules rather than being engaged in formation of an internal salt bridge. Exposure of these residues speaks in favor of their important role in tRNA binding.

Recently the ternary complex was crystallized in our lab, and structural studies have revealed that Lys-89 and Asn-90 together with Arg-288 constitute the primary tRNA binding site. The three residues act as the docking site for the tRNA 5' end. Structural analysis has shown that Arg-288 forms a salt bridge to the phosphate group of the 5' end, and Lys-89 and Asn-90 interact with the ribose of the 5' nucleotide of the tRNA (48).

Conserved amino acid specific residues involved in docking of the 3' CCA-Phe end are Glu-259, Val-225, and Ile-219. Finally the third region, the T stem, interacting with EF-Tu is conserved. The solvent-accessible surface area of His-118 has been measured to 0.5 Å\(^2\) in the GDP structure and 0 Å\(^2\) in that of GDPNP (11). His-118 is buried in the interface between domains 1 and 3 in both structural forms, and as the GTP conformation of EF-Tu is identical to the conformation of EF-Tu within the ternary complex, His-118 can hardly be directly involved in the binding of tRNA. The side chain of His-118 only forms hydrogen bonds to the backbone nitrogen of Gly-18 so even by structural examination an indirect role of His-118 in tRNA binding is still difficult to pinpoint.

As the functional role of His-118 is not obvious from inspection of established structures of EF-Tu, a functional role may be implicated when taking into account the dynamic properties of the enzyme. When EF-Tu undergoes its structural transformation, from the inactive GDP form to the tRNA binding GTP form, domains 1 and 3 have been suggested to dissociate temporarily (11). This would expose His-118 to the solvent and make it more likely to be involved in the structural transformation process, thus suggesting an indirect role of His-118 in tRNA binding.

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Elongation Factor Tu Mutants with Decreased tRNA Affinity