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EF-Tu PROTEIN DOMAINS
Functions and Thermostability

PhD thesis

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***Prohlášení**:*

Prohlašuji, že jsem nepředložila tuto disertační práci ani její podstatnou část k získání jiného nebo stejného akademického titulu.

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Abbreviations

aa residue	amino acid residue
aa-tRNA	aminoacyl-tRNA
B. stearothermophilus	*Bacillus stearothermophilus*
B. subtilis	*Bacillus subtilis*
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EF-G	elongation factor G
EF-Ts	elongation factor Ts
EF-Tu	elongation factor Tu
GAP	GTPase activating protein
GDP	guanosine 5'- diphosphate
GDPNP	guanosine 5'- (β, γ-imido)-triphosphate
GEF	guanine nucleotide exchange factor
GTP	guanosine 5'- triphosphate
PEP	phosphoenolpyruvate
PK	pyruvate kinase
S. solfataricus	*Sulfolobus solfataricus*
T. aquaticus	*Thermus aquaticus*
T. maritima	*Thermotoga maritima*
T. thermophilus	*Thermus thermophilus*
$\theta_{1/2}$ value	temperature of half inactivation of protein in GDP/GTP binding

Abstract

The bacterial elongation factor Tu (EF-Tu) has been extensively studied for decades as it plays a key role in protein biosynthesis. It is a model, multifunctional GTP-protein. This protein is also in the centre of interest as a possible target for new antibiotics. Moreover, the high homology in structure and function makes EF-Tu proteins suitable for the studies of evolutionary relationships between organisms and for elucidation of the structural features of adaptation to various living conditions. Furthermore, since all known EF-Tu proteins are composed of three distinct domains, they can also serve as suitable models for the understanding of domain organization in proteins.

One of the main research projects of the Department of Gene Expression at the Institute of Molecular Genetics AS CR, where I did my PhD studies, was the study of the primary structure, transcription regulation and functions of bacterial elongation factors Tu from Gram positive thermophilic bacterium *Bacillus stearothermophilus* and from Gram negative mesophilic bacterium *Escherichia coli*.

In this work, we focused on the structure-function relationships between EF-Tu proteins and their domains. The domain effect had been before our studies tested mainly by truncated EF-Tu forms lacking one or two domains. In contrast, we decided to study the properties of individual domains within full-length three-domain EF-Tu proteins (by domain chimerization approach) in comparison to isolated domain 1 (G-domain).

I focused on two topics:

1. Evaluation of the effect of individual domains of EF-Tu proteins from *E. coli* and *B. stearothermophilus* on their basic functions, namely GDP/GTP binding, GTPase activity and on the thermostability.

We showed that (i) *B. stearothermophilus* EF-Tu and *B. stearothermophilus* G-domain bound GDP and GTP with differential affinities in nanomolar and submicromolar ranges, respectively. These affinities were fully comparable with those of *E. coli* EF-Tu. In contrast, the *E. coli* G-domain did not display the differential affinity for GDP and GTP, typical for intact EF-Tus, and bound both nucleotides with much lower, micromolar affinities. Therefore, in *E. coli*, all three domains were required for the high and differential affinity for GDP and GTP, a physiological feature of bacterial EF-Tus. In contrast, the *B. stearothermophilus* G-domain itself already possessed the high and differential affinity for GDP and GTP.

(ii) The isolated catalytic G-domain of both EF-Tus displayed similar GTPase activities at their optimal temperatures. However, noncatalytic domains 2+3 of the EF-Tus influenced the GTPase activity of G-domains differently, depending on the domain origin. *E.*

coli domains 2+3 suppressed the GTPase activity of the *E. coli* G-domain, whereas those of *B. stearothermophilus* EF-Tu stimulated the *B. stearothermophilus* G-domain GTPase.

(iii) We demonstrated that the overall thermostability level of either EF-Tu was the result of cooperative interactions between the G-domain and domains 2+3. It appeared that the G-domains set up a basal level of the thermostability of both EF-Tus. Domains 2+3 contributed by further stabilization of α-helical regions of G-domains. This in turn, allowed the G-domains to function at temperatures corresponding to growth temperature optima of respective bacteria.

2. Characterization of thermostability elements of *B. stearothermophilus* G-domain by comparative analysis with the *B. subtilis* G-domain.

We demostrated that the N-terminal 12 amino acid residues play a key role in the thermostability of the G-domain. Our experiments further suggested that the thermostabilizing effect of the N-terminus could be mediated by stabilizing the functionally important effector region. The effect of the N-terminus was also significant for the thermostability of the full-length EF-Tu.

Our results contribute to the understanding of domain arrangement of ubiquitously occurring proteins elongation factors Tu. By a systematic analysis, we investigated the effect of the domains on EF-Tu functions and thermostability. The results obtained during my PhD studies were presented in five papers, in several oral presentations and in numerous posters at international conferences.

1. Introduction

This introduction is a brief description and not a comprehensive depiction of one of the most extensively characterized biological macromolecule elongation factor Tu (EF-Tu).

1.1 Elongation Factor Tu

The bacterial elongation factor EF-Tu is the most abundant protein in the bacterial cell (for a review see Kraal et al., 1999). It represents about 5-10% of all proteins and occurs at the ratio of 7-10 copies per ribosome (Zengel and Lindahl, 1990). The eukaryotic EF-1α counterpart is the second most abundant protein in the eukaryotic cell after actin (Condeelis, 1995).

EF-Tu is an essential protein and plays a crucial role in the elongation cycle of translation as a universal carrier of aa-tRNA from the cytosol of the cell to the A site on the ribosome (see chapter 1.2).

Bacterial elongation factors are highly conserved homologous proteins with high identity in the primary structure. They are composed of approximately 400 aa residues arranged into three distinct domains (see chapter 1.3). Their known 3-D structures are superposable and they share the same catalytic mechanisms (Krab and Parmeggiani, 1998; Jonak, 2007a).

EF-Tus belong to the group of GTP-proteins that play many essential roles in the cell such as signal transduction, protein biosynthesis, hormone response, neurotransmission, and cytoskeleton formation. Their conformation and activity are regulated by GDP and GTP (Kaziro, 1978; Printz and Miller, 1973; Jonak and Rychlik, 1973) and the proteins hydrolyze bound GTP (Krab and Parmeggiani, 1998).

EF-Tu interacts with many ligands, above all with guanine nucleotides, Mg^{2+} ion, aa-tRNA, ribosome and EF-Ts. EF-Tu is a target and its functions are influenced by four different types of antibiotics inhibiting protein synthesis (for a review see Hogg et al., 2002; Krab and Parmeggiani, 2002; Parmeggiani and Nissen, 2006): kirromycin, pulvomycin (Parmeggiani and Swart, 1985), GE2270 A (Anborgh and Parmeggiani, 1991) and enacyloxin IIa (Cetin et al., 1996). The inhibitory action of these antibiotics is based on two mechanisms. Kirromycin and enacyloxin IIa induce a constitutive activation of EF-Tu·GDP into a GTP-like conformation, making this complex stick to the mRNA-programmed ribosome after aa-tRNA binding and GTP hydrolysis, thus blocking the synthesis of a new peptide bond. Pulvomycin and GE2270 A prevent the formation of a stable ternary complex between EF-Tu·GTP and aa-tRNA (for a review see Hogg et al., 2002; Parmeggiani and Nissen, 2006).

The only one irreversible inhibitor of bacterial EF-Tu described is *N*-tosyl-L-phenylalanylchlormethylketone, which specifically reacts and modifies Cys81 in *E. coli* EF-Tu (Jonak et al., 1971; Jonak and Karas, 1989; Sedlacek et al., 1971; Jonak et al., 1982; Spirin et al., 1976).

Apart from the EF-Tu main function in protein biosynthesis, it has several other important functions. EF-Tu displays a proofreading activity during translation (Pape et al., 1999; Spahn and Nierhaus, 1998; Rodnina and Wintermeyer, 2001) (see chapter 1.2), represents the essential subunit of phage Qβ and SP1 RNA polymerase that produces phage RNA (Brown and Gold, 1996; Brown and Blumenthal, 1976; Mathu et al., 2003), and participates in the exclusion of T phages from several *E. coli* strains (Georgiou et al., 1998; Kraal et al., 1999). It is acetylated during nutrient deprivation (Arai et al., 1980; Jacobson and Rosenbusch, 1976) and associated with the outer membrane in both Gram positive (e.g. *Streptococcus pyogenes* (Rodriguez-Ortega et al., 2006)) and Gram negative (e.g. *Neisseria meningitidis* (Ferrari et al., 2006)) bacteria. Surprisingly, it was found that EF-Tu can also act as a molecular chaperone (Caldas et al., 1998; Malki et al., 2002). EF-Tu increases resistance of the ribosomal apparatus to antibiotics tetracycline, streptomycin, spectinomycin, erythromycin, and chloramphenicol (Spirin et al., 1976). A general feature is phosphorylation of EF-Tu during translation (Alexander et al., 1995). EF-Tu is also methylated during the exponential phase and mainly the stationary phase of the cell growth (for a review see Kraal et al., 1999). EF-Tu can also participate in the stringent response (Trigwell and Glass, 1998).

1.2 Elongation Factors in Protein Synthesis

The ribosomal elongation cycle of protein biosynthesis is one of the central processes of transformation of genetic information written in DNA into proteins. During the elongation cycle, the codon sequence of mRNA is translated into the aa sequence of a protein. The nascent polypeptide chain is extended by one aa residue at each cycle. Four elongation factors (two GTP proteins - EF-Tu and EF-G; EF-Ts and LepA) participate in the elongation cycle (Krab and Parmeggiani, 2002; Qin et al., 2006). Three models have been proposed for the elongation cycle – the allosteric three-site model, the hybrid state model and the α-ε model (for a review see Spahn and Nierhaus, 1998).

It was shown in *in vitro* conditions that the elongation cycle can be carried out by the ribosome itself, in the absence of elongation factors and GTP. Thus, the translation of mRNA using aa-tRNA is an inherent property of the ribosome. The elongation factors speed up the process, increase its accuracy and increase resistance of the system against some antibiotics and other chemicals (Spirin et al., 1976) (for a review see Wilson and Noller, 1998).

The analysis of tRNA binding on the ribosome revealed three sites - A, P and E. As the three tRNA-binding sites were detected on ribosomes from bacteria, archaea and eukarya, they were suggested to represent a universal feature of all ribosomes (for a review see Spahn and Nierhaus, 1998).

The elongation cycle can be divided into two parts - the EF-Tu cycle and the ribosome cycle (Fig. 1).

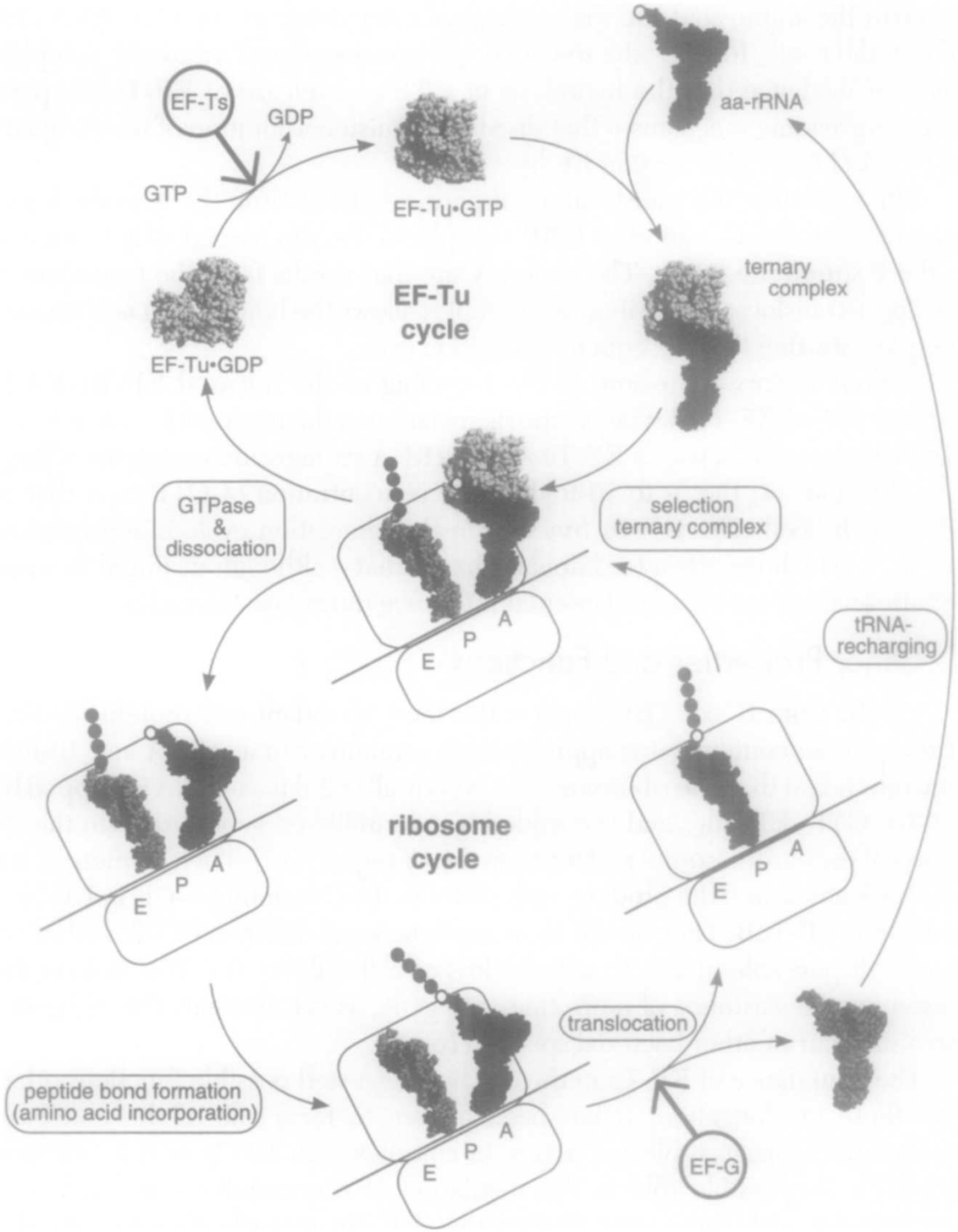

Fig. 1 Scheme of the elongation cycle of the protein biosynthesis. Adopted from (Krab and Parmeggiani, 2002).

During the **EF-Tu cycle**, the active EF-Tu·GTP binds aa-tRNA in the cytosol of the cell and delivers it to the A site on the ribosome. The A site contains the ribosomal decoding center that selects the correct (=cognate) aa-tRNA. The binding of aa-tRNA to the A site can be divided into two steps – tRNA selection (codon-anticodon recognition) and tRNA accommodation, separated by GTP hydrolysis. Two-step binding of aa-tRNA to the ribosome is important for maintaining the high accuracy of translation (EF-Tu proofreading activity). In the first step, the ternary complex forms a labile initial binding complex with the ribosome. Codon recognition triggers EF-Tu GTPase activation. Release of inorganic phosphate induces an extensive conformational transition of EF-Tu from the GTP- to the GDP-bound form, whereby the factor loses the affinity for aa-tRNA and dissociates from the ribosome. Then, the aa-tRNA released from EF-Tu is free to move into the 50S A site. The 3′ end of tRNA is accommodated in the peptidyl-transferase centre where it takes part in rapid peptide bond formation. Binding of the cognate tRNA to the A site is strongly stabilized and GTP hydrolysis by EF-Tu is strongly stimulated, whereas binding of the near-cognate codon is weak and GTP hydrolysis slow. It enables dissociation of the near-cognate aa-tRNA from the ribosome. For non-cognate ternary complexes, there is no significant codon-anticodon interaction and non-cognate ternary complexes are rejected at the initial selection stage prior to GTP hydrolysis. After binding of the aa-tRNA to the A site on the ribosome, the discharged tRNA from the preceding elongation cycle is released from the ribosomal E site (for a review see Daviter et al., 2003; Ogle and Ramakrishnan, 2005; Spahn and Nierhaus, 1998; Jonak, 2007b). The inactive EF-Tu·GDP is activated by the elongation factor Ts (EF-Ts) that belongs to the family of the GEF (guanine-nucleotide exchange factors) (Dahl et al., 2006; Kawashima et al., 1996; Schummer et al., 2007; Wang et al., 1997).

The first step of the **ribosome cycle** is peptide-bond formation between the aa-tRNA in the A site and peptidyl-tRNA in the P site. The peptidyl-transferase reaction is the feature of 50S ribosomal subunit resulting in the peptidyl-tRNA located in the A site and the discharged tRNA in the P site (PRE state of the ribosome). The next step of the ribosomal cycle is the translocation of peptidyl-tRNA from the A to the P site and movement of mRNA by one codon. After translocation, the ribosome is occupied by the peptidyl-tRNA in the P site and discharged tRNA in the E site, and the next elongation cycle can start by binding of new ternary complex to the A site (POST state of ribosome). The translocation is catalyzed by the third elongation factor EF-G that reduces the activation energy barrier between the ribosomal PRE and POST states. Hydrolysis of GTP bound to the EF-G is necessary for this reaction (Spahn and Nierhaus, 1998; Andersen et al., 2003).

Recently, it was demonstrated that the extremely conserved LepA protein, present in all bacteria and mitochondria, is required for accurate and efficient protein synthesis. LepA

was proposed to have a unique function in back-translocating posttranslocational ribosomes: it recognizes the ribosome after a defective translocation reaction and induces back-translocation, thus giving EF-G a second chance to translocate the tRNA correctly. LepA was proposed to be the fourth elongation factor (Evans et al., 2008; Qin et al., 2006).

Comparison of the 3D structures of the EF-G in GDP form and the EF-Tu·GTP·aa-tRNA ternary complex showed a high similarity between both structures (Fig. 2) and the theory about the molecular mimicry was proposed. The hypothesis assumes that EF-G·GDP after leaving the ribosome imprints its structure into the ribosome and the ternary complex binds in the same binding site. This phenomenon is expected to be more general during the translation and analogous situations were proposed for complexes of initiation and termination factors (Clark and Nyborg, 1997; Nyborg et al., 1996; Nyborg et al., 1997; Nyborg and Liljas, 1998; Kristensen et al., 2002).

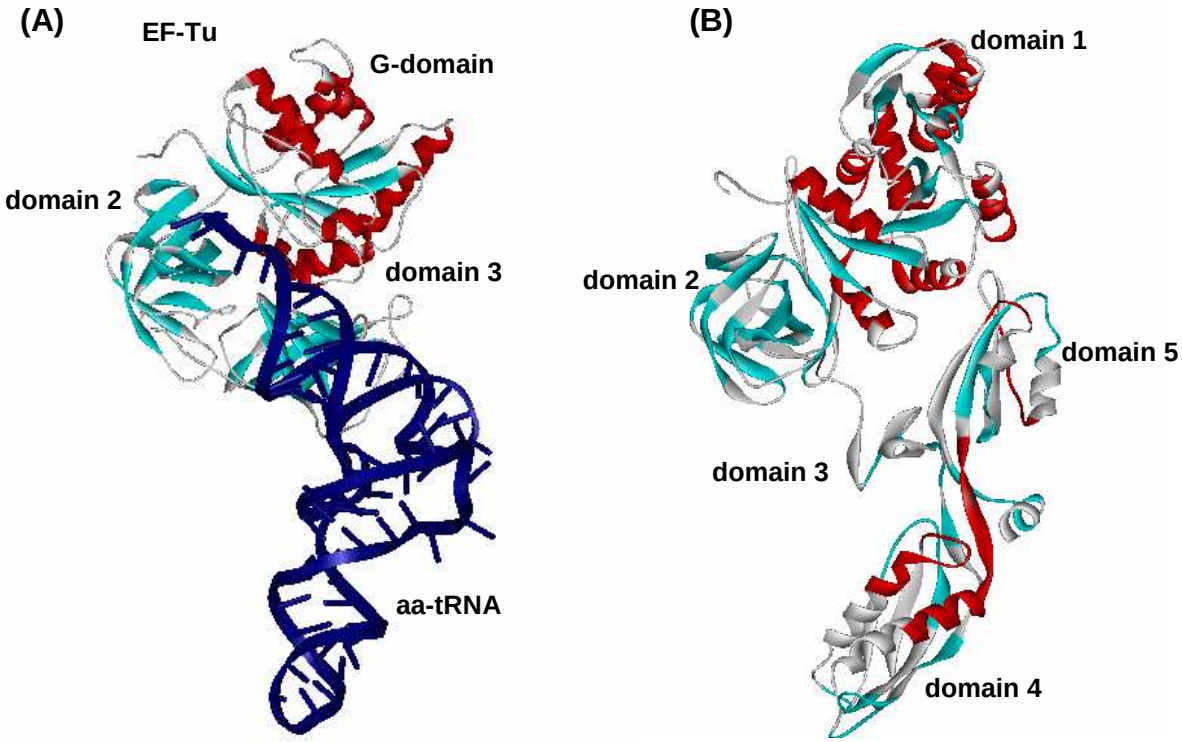

Fig. 2 Molecular mimicry between the EF-Tu·GDPNP·aa-tRNA ternary complex (A) and EF-G·GDP (B). Adopted from (Nyborg et al., 1996).

1.3 EF-Tu Structure

The 3D structures of EF-Tu in complexes with guanine nucleotides either GDP from *E. coli* (Abel et al., 1996; Song et al., 1999; Polekhina et al., 1996) and *Thermus thermophilus* (Polekhina et al., 1996) or a non-hydrolyzable GTP analogue GDPNP from *Thermus thermophilus* (Berchtold et al., 1993) and *Thermus aquaticus* (Kjeldgaard et al., 1993) have been solved. They showed a high degree of similarity in the EF-Tu structure and

discovered a significant difference between the active GTP and inactive GDP conformations of EF-Tu.

About 400 aa residues of EF-Tu are arranged into three distinct domains (Fig. 3). The N-terminal half of the molecule (aa residues ~1-200) represents domain 1, usually called the G-domain as it is the catalytic part of the molecule binding GDP and GTP nucleotides. The G-domain has a tertiary structure closely resembling that of Ras-p21. The G-domain is arranged into the central β-sheet of five parallel and one antiparallel strands surrounded by six to seven α-helices and 10 loop-like segments connecting the elements of secondary structure (Berchtold et al., 1993).

Domains 2 and 3 are both barrel-shaped (approx. 100 aa residues each), consisting mainly from antiparallel β-strands. Both domains are closely associated by hydrogen bonds and polar interactions. Domain 2 (aa residues ~210-300) is arranged into a classical six-stranded Greek key structural motif. Domain 3 (aa residues ~301-400) is a six-stranded jelly roll. Domain 3 is stabilized by a compact hydrophobic core, consisting of six aromatic side chains (Kjeldgaard et al., 1993).

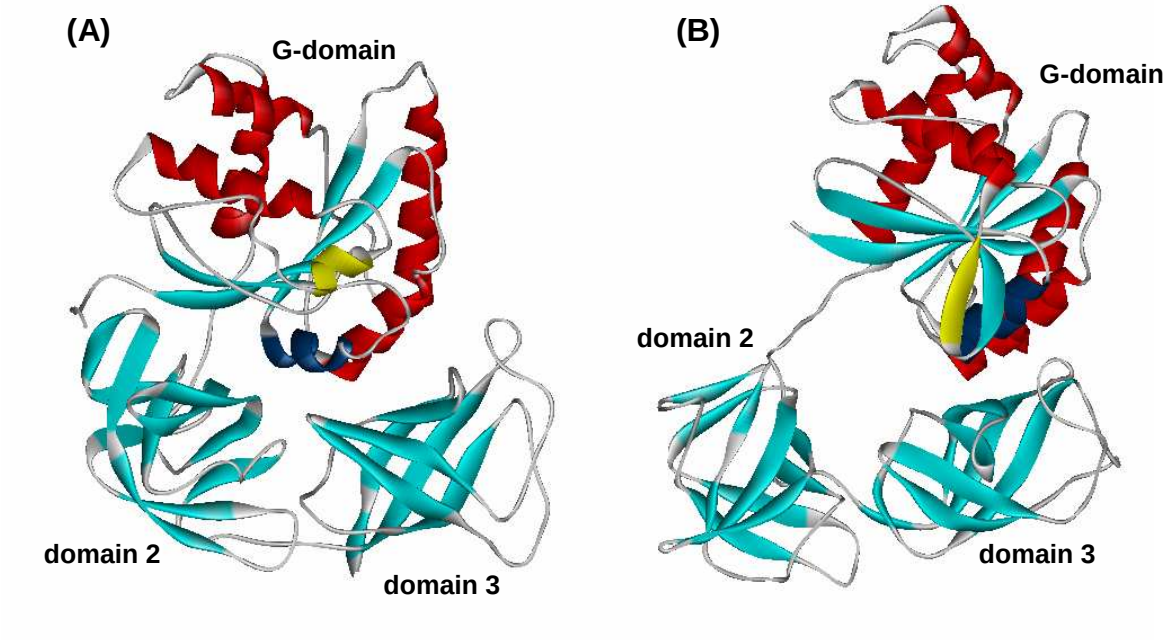

Fig. 3 3D structure of *T. aquaticus* EF-Tu in "active" GTP (A) and "inactive" GDP (B) conformation. Two parts of the G-domain that markedly change their positions and conformation after GTP hydrolysis are highlighted – the α-helix B is shown in dark blue; the α-helix A´´ that is unwound into β-strand b´ is in yellow.

G-domain – Nucleotide and Mg^{2+} Binding

The G-domains contain several specific structural regions important for ligand binding and conformational changes between GDP and GTP forms of EF-Tu proteins.

The nucleotide binding site is situated in a hydrophobic pocket on the surface of the G-domain (Kjeldgaard et al., 1996). It contains three conserved consensus sequences typical for all GTP-binding proteins mainly involved in the nucleotide binding and one additional motif characteristic for EF-Tus (Fig. 4). Motif I (GxxxxGKS/T, residues 18-25 in *E. coli* EF-Tu) is usually called P-loop or phosphate-binding loop or glycine-rich loop. It is wrapped around the β-phosphate group. Motif II (DxxG, residues 80-83) is situated close to the P-loop and is in contact with it. It also contacts the bound Mg^{2+} ion and is involved in the conformational change induced by GTP hydrolysis (see below). Motif III (NKxD, residues 135-138) is a guanine base recognition element and it determines the specificity for guanine. Motif IV that was found within EF-Tu proteins (SALx, residues 173-176) coordinates the guanine base (Kjeldgaard et al., 1996; Krab and Parmeggiani, 2002; Hwang and Miller, 1987; Krab and Parmeggiani, 2002; Weijland and Parmeggiani, 1993).

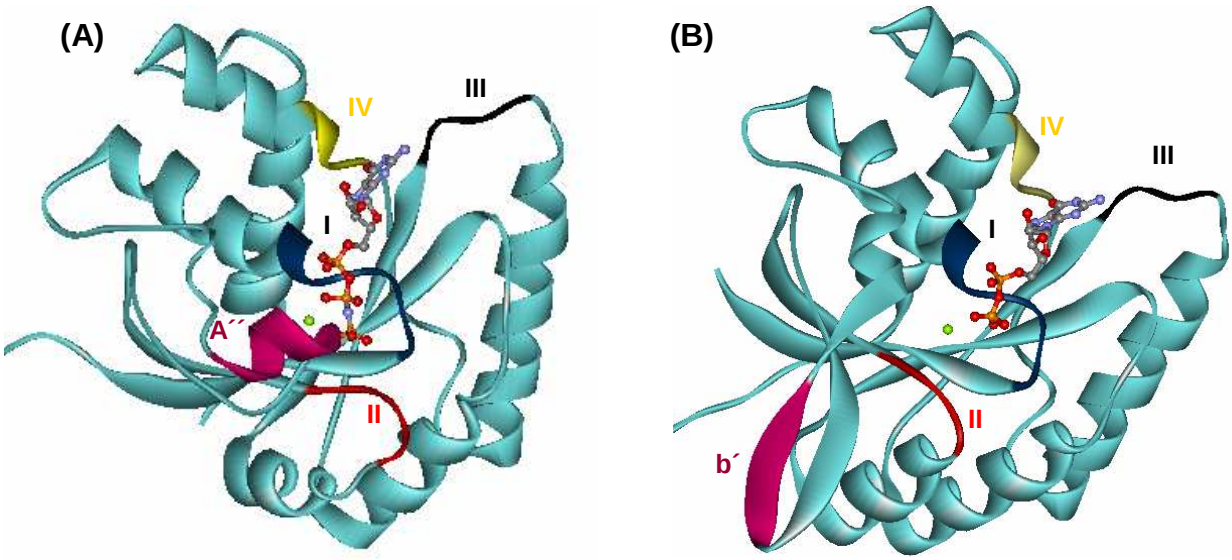

Fig. 4 3D structure of the *T. aquaticus* G-domain in the GTP (A) and GDP (B) form. The four consensus sequences are highlighted. Conserved motifs are colored (motif I in dark blue; motif II in red; motif III in black; motif IV in yellow). The unwinding of α-helix A´´ into β-list b´ triggered by GTP hydrolysis is shown (purple). Ion Mg^{2+} is shown in green and nucleotides in ball and stick model.

G-domains, furthermore, contain two regions (switch I and switch II) important for the conformational change between active GTP and inactive GDP form of the protein. The switch I region (residues 40-62) is also called “effector region” by the analogy with Ras-p21 (Kjeldgaard et al., 1993; Kjeldgaard et al., 1996). It can be divided into two parts – the N-terminal part of switch I is the most variable region within bacterial EF-Tus and, conversely, the C-terminal part of switch I is well conserved among prokaryotic factors. Switch I forms the vicinity of the magnesium ion and through the water molecule interacts with it. This part

undergoes dramatic change after GTP hydrolysis (see below) (Abel et al., 1996; Berchtold et al., 1993; Kjeldgaard et al., 1993; Kjeldgaard et al., 1996; Polekhina et al., 1996; Song et al., 1999).

The switch II region is represented by α-helix B and its surroundings. It contains motif II (Kjeldgaard et al., 1996).

The Mg^{2+} ion is an essential cofactor for binding of guanine nucleotides and GTPase reaction in all guanine nucleotide binding proteins (Kjeldgaard et al., 1996). It is positioned in a deep cleft in the protein, separating the nucleotide binding pocket and the switch regions. The Mg^{2+} ion ties together the four parts of the G-domain – the nucleotide binding site, the switch I region (effector region), the switch II region and the nucleotide cofactor (Kjeldgaard et al., 1993; Kjeldgaard et al., 1996).

GTP Hydrolysis

The importance of GTP hydrolysis for the EF-Tu physiological activities was already recognized in 1960s. Despite many studies, the mechanism of this reaction in EF-Tu proteins remains in large part unknown (for a review see Krab and Parmeggiani, 2002).

The activation of hydrolysis of GTP bound to EF-Tu is triggered by codon-anticodon recognition. tRNA plays an active role in communicating the trigger signal from the decoding site to the functional sites on the 50S subunit to accelerate GTP hydrolysis and subsequent tRNA accommodation**.** A single residue in the G-domain of EF-Tu, His84 in the switch II region of EF-Tu, is directly involved in the chemistry step of the reaction. In the GTP-bound form of EF-Tu, the catalytic His is turned away and shielded from the γ-phosphate by several hydrophobic residues, called the hydrophobic gate, precluding access of the catalytic His residue to the nucleotide binding pocket. GTPase activation is likely to involve a rearrangement of the G-domain of EF-Tu, which can be envisaged as opening of the hydrophobic gate and reorientation of His84 towards the catalytic site. The neighboring residue Gly83 of EF-Tu plays an important role in both the rearrangement of the switch II region upon GTPase activation, due to the conformational flexibility inherent to Gly residues, and in the GTP hydrolysis itself, probably by helping to position the catalytic water by hydrogen bonding with the main chain oxygen of Gly83. Several ribosomal elements (mainly ribosomal proteins L7/12 and the α-sarcin loop of 23S rRNA) may contribute to the enhancement of GTPase activation as well. Conformational changes of the 30S subunit in response to codon recognition are also important (for a review see Daviter et al., 2003).

EF-Tu – Mechanism of Domain Rearrangement

It has been proposed that three domains of EF-Tu work as two rigid moieties – the G-domain as a "head" and domains 2+3 with a tight interface as a "tail" (Kjeldgaard et al., 1993).

In the GTP conformation, the EF-Tu is arranged into a compact globular shape with tight interfaces between all three domains (Fig. 3). This form displays high affinity for aa-tRNA and ribosomes (Berchtold et al., 1993; Kjeldgaard et al., 1993).

After the GTP hydrolysis to GDP and inorganic phosphate, the conformation of the molecule is dramatically changed (Abel et al., 1996; Polekhina et al., 1996). Removal of the γ-phosphate affects both switch regions. The disruption of the hydrogen bond between Gly83 and γ-phosphate causes the flip of peptide bond between Pro82 and Gly83 by about 150° resulting in formation of a new bond between Pro82 and β-phosphate. The change in the localization of the peptide bond influences the position of α-helix B – the last C-terminal turn is unwound and a new turn is formed at the N-terminus of this helix. Furthermore, the reorientation of Pro82 causes local changes in the switch I region. After the shift of peptide bond Ile62-Asn63, the upstream 12 aa residue chain is reoriented and changes its structure - the short α-helix A´´ is unwound into β-strand b´ (Figs. 3, 4) (Abel et al., 1996; Polekhina et al., 1996).

Local changes within the switch regions result in extensive conformational changes of the whole EF-Tu. The axis of α-helix B is reoriented by 42°. This induces loss of the interdomain interactions and rotation of G-domain by 90° relative to domains 2+3, with formation of a new G-domain-domain 3 interface. The G-domain-domain 2 interactions are lost and these domains become separated by the opening of the molecule (Fig. 3). Some parts of the molecule are shifted by about 40Å. The open EF-Tu·GDP form loses affinity for aa-tRNA and ribosomes (Abel et al., 1996; Kjeldgaard et al., 1993).

Valuable information about the 3D structure and function of EF-Tu can also be obtained from 3D structures of EF-Tu·GDPNP·aa-tRNA ternary complexes (Nissen et al., 1995; Nissen et al., 1999), complexes of EF-Tu and EF-Ts (Kawashima et al., 1996; Wang et al., 1997) and complexes of EF-Tu with different antibiotics (for a review see Hogg et al., 2002; Parmeggiani and Nissen, 2006).

1.4 Domain Functions and Thermostability of EF-Tu

EF-Tus are multifunctional three-domain proteins. They serve as a perfect model for elucidation of the question: How are the functions and properties of such proteins built from the contributions of their domains?

This chapter depicts observations describing the effects of individual domains on basic EF-Tu functions (GDP/GTP binding and GTPase activity) and on thermostability.

The role of individual domains has been studied by two approaches: (i) using truncated EF-Tus, lacking one or two domains, and (ii) using chimeric proteins prepared by swapping of individual domains between two different EF-Tus. This method enables us to evaluate domain functions within the three-domain structure. Methods and procedures for isolation and purification of bacterial EF-Tus, their mutants, chimeric forms and G-domains are summarized in (Jonak, 2007a).

Truncated EF-Tus

A prominent role in the investigation of EF-Tu functions is played by this protein from *E. coli*. It was the G-domain of EF-Tu from this organism, which was the first one that was prepared separately and which was functionally investigated (Parmeggiani et al., 1987). This characterization identified the G-domain as a discrete functional module of EF-Tu. The isolated G-domain was found to retain basic functions of EF-Tu such as GDP/GTP binding and GTPase activity. Differences between the *E. coli* G-domain and the intact *E. coli* EF-Tu involve the loss of differential affinity for guanine nucleotides, the inability to bind aa-tRNA and a strong decrease in affinity for GDP. The physiological 100x higher affinity of *E. coli* EF-Tu for GDP than for GTP is a typical feature of bacterial EF-Tus (Anborgh et al., 1992). On the other hand, the *E. coli* G-domain displayed similar K'_d values in both complexes with GDP and GTP, the affinity for GDP was 1000x, and for GTP 10x lower, in comparison with *E. coli* EF-Tu (Parmeggiani et al., 1987). Interestingly, the same loss in affinity for GDP was also displayed by the truncated *E. coli* EF-TuΔ3 (lacking the domain 3) and EF-TuΔ2 (lacking the domain 2). Their binding affinities were similar to those obtained for the G-domain. Thus, a typical feature of *E. coli* EF-Tu, the differential affinity for GTP and GDP, results from a cooperation involving all three domains (Cetin et al., 1998). The loss of the affinity for GDP was also determined for *T. thermophilus* G-domain (Nock et al., 1995).

The intrinsic GTPase activity of *E. coli* G-domain was even higher than that of *E. coli* EF-Tu. On the other hand, The GTPase activity of *E. coli* truncated EF-Tu forms, EF-TuΔ3 and EF-TuΔ2, were not markedly influenced by the truncation (Cetin et al., 1998; Jensen et al., 1989; Parmeggiani et al., 1987). Surprisingly, no stimulatory effect of ribosomes on the GTPase activity of both truncated forms was observed (Cetin et al., 1998), although the ribosomes slightly enhanced the GTPase activity of the isolated *E. coli* G-domain (Jensen et al., 1989; Parmeggiani et al., 1987). The intrinsic *E. coli* GTPase seemed to be the main function of the G-domain; nevertheless, for its optimal stimulation by ribosomes the presence of all three domains was needed.

A different situation was observed with *T. thermophilus* EF-Tu. The G-domain displayed about half of the intrinsic GTPase activity and EF-TuΔ3 a 39-fold increased rate of GTPase as compared to the full-length protein. The ribosomes stimulated the G-domain

GTPase 3-fold less than intact EF-Tu and were not able to stimulate GTPase of EF-TuΔ3. These results suggested the GTPase-activating role of domain 2 and an inhibitory effect of domain 3 in the *T. thermophilus* EF-Tu (Nock et al., 1995).

Thermostabilities of EF-Tu proteins have been mainly measured in functional tests, by the ability to retain GDP/GTP binding activity at increasing temperature, and $\theta_{1/2}$ values (temperatures of half inactivation of their GDP/GTP binding activities) were determined. Comparison of thermostabilities of *E. coli* EF-Tu in GDP and GTP form showed unexpected results. The open EF-Tu·GDP form was by about 8℃ more thermostable than the compact GTP form. No structural explanation for this phenomenon was found (Anborgh et al., 1992). Analysis of stabilities of *E. coli* truncated forms in GDP forms showed profound loss of the thermostability of *E. coli* G-domain ($\theta_{1/2}$=37℃), in comparison with the respective EF-Tu ($\theta_{1/2}$=51℃). The *E. coli* EF-TuΔ3 displayed a similar thermostability as the G-domain, since in the GDP form no interaction between G-domain and domain 2 that could stabilize the G-domain has been detected. On the other hand, the *E. coli* EF-TuΔ2 was only slightly less thermostable than intact EF-Tu, suggesting a stabilization effect of domain 3 on the full-length EF-Tu (Cetin et al., 1998).

Interestingly, thermostability of the *T. thermophilus* G-domain in GDP form ($\theta_{1/2}$=39℃) was almost the same as that of *E. coli* G-domain. It was far less thermostable than *T. thermophilus* EF-Tu ($\theta_{1/2}$=88℃). Furthermore, the domain 2 within the *T. thermophilus* EF-TuΔ3 remarkably stabilized the G-domain and this form was only by about 10℃ less thermostable than EF-Tu. This is not easily to reconcile with available 3D structures of EF-Tu·GDP proteins. However, it may suggest the existence of an artificial G-domain-domain 2 interaction in GDP form of this truncated EF-TuΔ3.

The thermal stability of EF-Tu/EF-1α was evaluated in two hyperthermophilic organisms *T. maritima* (Sanangelantoni et al., 1996) and archaea *S. solfataricus* (Masullo et al., 1997). Both hyperthermophilic G-domains were in the GDP form by about 10℃ less thermostable than the respective EF-Tu/EF-1α. Since both G-domains still displayed high thermostability, they obviously contain some thermostabilizing elements. The removal of only domain 3 in the *S. solfataricus* EF-1αΔ3 caused the same decrease in the thermostability in the GDP form as with the G-domain, indicating no stabilizing effect and no interaction between the G-domain and domain 2 (Masullo et al., 1997).

Chimeric EF-Tus

Three-domain chimeric forms of EF-Tu/EF-1α were investigated from both hyperthermophilic organisms mentioned above. The chimera containing the *S. solfataricus* G-domain and *E. coli* domains 2+3 was in the GDP form ($\theta_{1/2}$=82℃) less thermostable than *S. solfataricus* EF-1α ($\theta_{1/2}$=96℃) and also slightly less thermostable than isolated *S.*

solfataricus G-domain ($\theta_{1/2}$=86℃), confirming the presence of thermostabilizing elements within the G-domain and no stabilizing effect of *E. coli* domains 2+3 on *S. solfataricus* G-domain (Arcari et al., 1999).

In the case of *T. maritima* EF-Tu, two chimeras were characterized: the EF-Tu and the G-domain with N-terminal 90 aa residues of the G-domain substituted by the same region from *E. coli* EF-Tu. Both chimeric forms were markedly less thermostable than the original proteins. The N-terminal 90 residues were proposed to play an important role in thermostabilizing *T. maritima* EF-Tu. However, the opposite chimeric EF-Tu containing the N-terminal 90 aa residues from *T. maritima* EF-Tu and the rest of the molecule from *E. coli* did not result in increased stability. It has been suggested that the context of unique interaction between certain N-terminal residues and rest of the molecule of *T. maritima* EF-Tu are necessary to attain the overall thermostability of *T. maritima* EF-Tu (Sanangelantoni et al., 1996).

1.5 Thermostability of Proteins

Thermostability of proteins has been a widely studied topic for many years. Thermophilic proteins attract increasing attention owing to their potential utilization in science (DNA polymerases, DNA ligases, proteases) and industry (proteases as additives to detergents; α-amylases, glucose and xylose isomerases in the starch-processing industry; various enzymes in organic synthesis, waste treatment, diagnostics, etc.) (for a review see Vieille and Zeikus, 2001).

Thermophilic proteins belong to extremozymes, proteins-enzymes isolated from extremophiles, microorganisms living in the environment of extreme conditions, e.g. low (-2 to 15℃) and high (60-110℃) temperatures, a high ionic strength (2-5 M NaCl) and extreme pH (<4, >9) (Hough and Danson, 1999). The expression of almost all thermophilic proteins in a mesophilic organism without loss of their activity and thermostability facilitates their examination (Burdette et al., 1996; Tomschy et al., 1993; Vieille et al., 1995; Zwickl et al., 1990). Thermophilic and mesophilic proteins hold a high similarity. Their 3-D structures are superposable; they share the same catalytic mechanisms, and their amino acid sequences are similar in 40-85% (Burdette et al., 1996; DiRuggiero et al., 1993; Fujinaga et al., 1993; Vieille et al., 1995; Vieille and Zeikus, 2001). The fact that a thermophilic protein is stable and active at a higher temperature is, therefore, written in its amino acid sequence (Vieille and Zeikus, 1996) and should be elucidated from differences in sequences between mesophilic and thermophilic counterparts. While most thermophilic enzymes are intrinsically stable, some intracellular enzymes get their high thermostability from intracellular factors such as salts, high protein concentrations, coenzymes, substrates, or activators of general stabilizers such as thermamine (for a review see Vieille and Zeikus, 2001).

Differences in ΔG_{stab} as small as 3 to 6.5 kcal/mol were reported to account for thermostability increases between 10-20℃. This indicates that, in principle, only few changes in non-covalent forces responsible for ΔG_{stab} can distinguish e. g. mesophilic from thermophilic proteins (Vieille and Zeikus, 2001).

Different thermophilic proteins adapt to higher temperatures by different sets of structural devices. Several rules were suggested for thermostabilization of thermophilic proteins (for a review see Vieille and Zeikus, 2001; Zhou et al., 2008). Thermophilic proteins can exhibit higher core hydrophobicity (Pace, 1992; Pace et al., 1996; Pape et al., 1999; Schumann et al., 1993), greater numbers of ionic interactions (Cambillau and Claverie, 2000; Suhre and Claverie, 2003; Vetriani et al., 1998), increased packing density (Russell et al., 1997), additional networks of hydrogen bonds (Jaenicke and Bohm, 1998), decreased lengths of surface loops (Thompson and Eisenberg, 1999), stabilization by heatstable chaperones (Haslbeck et al., 2005), an increase in disulfide bond formation (Beeby et al., 2005) and a general shortening of length (Tekaia et al., 2002).

Hyperthermophiles (growth optimum 90-110℃) and thermophiles (growth optimum 45-80℃) utilize various adaptive strategies in stabilization at an increased temperature (Szilagyi and Zavodszky, 2000; Li et al., 2005; Szilagyi and Zavodszky, 2000). Moderately thermophilic proteins display a significant increase in the polarity of exposed surfaces, a higher number of weaker ion pairs, an increase of α-helices, replacements of Lys by Arg and a decreased amount of Ser. Conversely, hyperthermophilic proteins have extra strong ion pairs, a prevalent increase in β-strands, a decrease in thermochemically instable Met and Asp, and a decreased number of internal cavities. Comparison of mesophilic and thermophilic protein structures indicate that the hydrophobic effect has a higher contribution to stability at higher temperatures. However, in comparison with salt bridges, the hydrophobic interactions are broken at high temperatures (Szilagyi and Zavodszky, 2000). Therefore, salt bridges are significant in stabilizing proteins at a high temperature (Elcock, 1998).

Several experimental approaches are being used to study thermostability of proteins:

- site-directed mutagenesis and substitution of amino acids
- creation of chimeric proteins from thermophilic and mesophilic counterparts; creation of truncated forms of proteins
- random mutagenesis of proteins (e.g. Hancock et al., 2006); error-prone PCR (Cadwell and Joyce, 1994).
- comparison of three-dimensional structures of mesophilic and thermophilic proteins (e.g.Melchionna et al., 2006)
- statistical techniques that compare patterns of pairs of amino acid substitutions in mesophilic and thermophilic proteins (e.g. Cambillau and Claverie, 2000; Suhre and Claverie, 2003; Szilagyi and Zavodszky, 2000; McDonald et al., 1999).

To date, the satisfactory understanding of the origins of adaptation at extreme conditions remains elusive. The complexity of each protein structure prevents the definition of universal stabilization mechanisms and each thermostable protein is stabilized by a unique combination of different mechanisms (Jaenicke and Bohm, 1998; Li et al., 2005; Scandurra et al., 1998; Vieille and Zeikus, 1996; Zhou et al., 2008).

2. Aims of the Work

At the beginning of my PhD studies, functional evaluation of individual domains of EF-Tus had been carried out mainly by the deletion approach: by examining one- or two-domain proteins (Cetin et al., 1998; Masullo et al., 1997; Nock et al., 1995; Parmeggiani et al., 1987). Here we used a chimerization approach. We swapped individual domains between EF-Tus from two different organisms. This method allowed us to study the domain effects within full-length, three-domain EF-Tu.

We addressed (i) how the functions and thermostability of EF-Tus from mesophilic *Escherichia coli* and thermophilic *Bacillus stearothermophilus* depend on individual domains, and (ii) what elements of the isolated domain 1 (G-domain) confer thermostability to this functional module of EF-Tu, using mesophilic *B. subtilis* and thermophilic *B. stearothermophilus* as model organisms. Our research can be divided into three parts, outlined below.

1. Preparation and purification of EF-Tu proteins, their G-domains, and their mutant and chimeric forms.

- Construction of chimeric genes for chimeric EF-Tus and preparation of expression vectors containing genes for EF-Tus, their G-domains, and chimeras.
- Optimization of overexpression and purification of EF-Tu forms to obtain sufficient amounts of proteins in high yield and purity for functional tests and structural studies.

2. Analysis of domain contribution to EF-Tu functions and thermostability

- Comparative analysis of the properties of EF-Tus from mesophilic *E. coli* and thermophilic *B. stearothermophilus*, their G-domains and chimeric EF-Tus composed of swapped domains.

3. Investigation of thermostability elements in *B. stearothermophilus* G-domain

- Identification of thermostability elements in *B. stearothermophilus* G-domain by a systematic swapping approach of protein regions differing between this G-domain and mesophilic *B. subtilis* G-domain.

3. Results and Discussion

1. Preparation and purification of EF-Tu proteins, their G-domains, and their mutant and chimeric forms.

(i) Genes for EF-Tu proteins and G-domains were prepared using PCR. Genes for the site-directed mutants were obtained by QuikChange Site Directed Mutagenesis adopted from Stratagene. The PCR technology was also successfully used to construct chimeric genes in three steps (Fig. 5). First, the individual fragments for the construction of chimeric genes were amplified separately using primers containing two parts – the 5´ flanking region (Fig. 5; primer C red part) coding for a domain from one EF-Tu and the 3´ region (Fig. 5; primer C blue part) coding for the next domain from another EF-Tu. Second, the corresponding fragments were annealed (using ends overlapping the regions of different origin) and then the whole chimeric genes were synthesized. Finally, the whole gene was amplified using end primers.

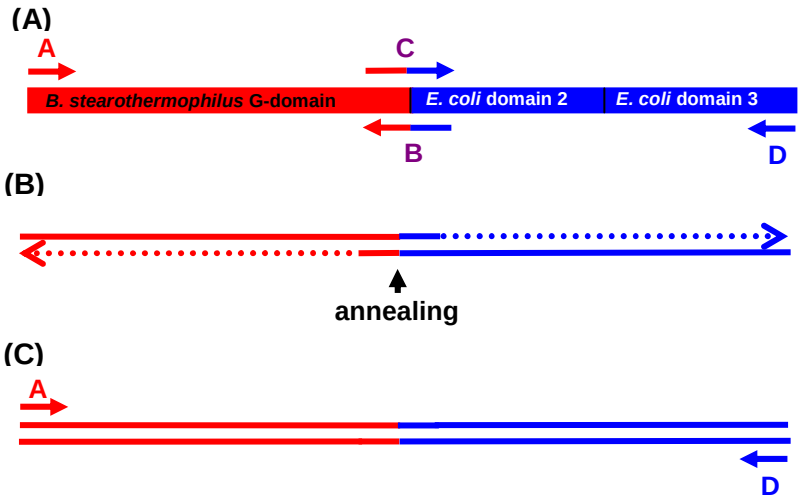

Fig. 5 Scheme of constructing a chimeric gene. (A) PCR synthesis of two fragments of a chimeric gene by primers A+B and C+D is indicated. (B) Annealing of both fragments and synthesis of whole chimeric gene. (C) Final amplification of chimeric gene by primers A+D.

(ii) Expression vectors pGEX-5X-3, pGEX-1 and pFLAG-CTC and *E. coli* strain BL21 were used for overexpression of proteins.

(iii) Affinity chromatography based on the GST technology was adopted and optimized for purification of EF-Tu forms. It was necessary to modify the washing step of Glutathione Sepharose 4B bound fusion protein, in particular, to obtain proteins in high purity. The removal of GST moiety from the recombinant proteins by proteolytic cleavage by

factor Xa required to design a reliable procedure to prevent degradation/cleavage of *B. sterothermohilus* EF-Tu and proteins containing *B. sterothermophilus* G-domain.

A reliable method for the preparation of chimeric genes was established. Several modifications in the standard isolation and purification procedures by GST technology were introduced that proved necessary to obtain the proteins in a purified and undegraded form. *(Paper #1 – Tomincová et al., 2002).*

2. Analysis of domain contribution to EF-Tu functions and thermostability

Two EF-Tu proteins from mesophilic *E. coli* (optimal growth temperature 37℃) and thermophilic *B. stearothermophilus* (optimal growth temperature 58℃) were investigate d.

Six chimeric EF-Tu proteins containing all combinations of domains of EF-Tu from *E. coli* and *B. stearothermophilus*, recombinant *E. coli* and *B. stearothermophilus* EF-Tus and respective G-domains were purified and analyzed. First, isolated proteins were tested in GDP and GTP binding. k_{-1}, k_{+1}, K_d constants for GDP and GTP were determined or calculated. Second, the intrinsic GTPase activities of investigated proteins were measured. Third, GDP/GTP binding experiments and GTPase activity measurements were carried out at increasing temperature and the thermostabilities of proteins were determined. Fourth, the thermostabilities of proteins were also determined by direct measuring of α-helix stability at increasing temperature using CD spectroscopy at 220 nm. Finaly, structural mechanisms of thermostabilization of *B. stearothermophilus* G-domain were suggested.

We demonstrated that (i) *B. stearothermophilus* EF-Tu and *B. stearothermophilus* G-domain bound GDP and GTP with affinities in nanomolar and submicromolar ranges, respectively, fully comparable with those of *E. coli* EF-Tu. In contrast, the *E. coli* G-domain bound the nucleotides with much lower, micromolar affinities, as was also shown by Parmeggiani and coworkers (1987) . The *E. coli* G-domain required the presence of domains 2+3 for the differential physiological affinity for GDP and GTP of *E. coli* EF-Tu. On the other hand, the *B. stearothermophilus* G-domain by itself already possessed the differential physiological affinity for GDP and GTP. The exchange of domains 2 and 3 had essentially no effect on the GDP binding activity; all complexes of chimeric EF-Tus displayed high affinity for GDP with K_ds in the nanomolar range.

(ii) The isolated catalytic G-domains of both EF-Tus displayed similar GTPase activities at their optimal temperatures. However, noncatalytic domains 2+3 of the EF-Tus influenced the GTPase activities of G-domains differently, depending on the domain origin. *E. coli* domains 2+3 suppressed the GTPase activity of the *E. coli* G-domain, whereas those of *B. stearothermophilus* EF-Tu stimulated the *B. stearothermophilus* G-domain GTPase.

(iii) The final thermostability level of either EF-Tu was the result of a cooperative interaction between the G-domains and domains 2+3. The G-domains set up a basal level of

the thermostability, which was about 20℃ higher with the *B. stearothermophilus* G-domain than with the *E. coli* G-domain. This correlated with the growth temperature optimum difference of both bacteria. Two distinct thermostabilization features of the *B. stearothermophilus* G-domain were found: an increase of charged residues at the expense of polar uncharged residues (*CvP* bias), and a decrease in the nonpolar solvent accessible surface area. Domains 2+3 contributed by further stabilization of α-helical regions of G-domains. This in turn allowed the G-domains to function at temperatures corresponding to growth temperature optima of the respective bacteria. The contributions of domains 2+3 were similar, irrespective of their origin. However, with *E. coli* domains 2+3 they depended on the guanine nucleotide binding state: the stabilization effect of domain 2+3 was lower in the GTP conformation than in the GDP conformation, and the mechanism involved destabilization of the α-helical regions of the G-domain by *E. coli* domain 2.

The presented results provide evidence for similar and different roles of the non-catalytic domains in the regulation of functions and thermostability of the two EF-Tu proteins. *(Paper #2 – Šanderová et al., 2004, Paper #3 – Šanderová and Jonák, 2005).*

3. Investigation of thermostability elements in *B. stearothermophilus* G-domain

This project was based on our previous results showing that the G-domains set up the basal level of the thermostability of *E. coli* and *B. stearothermophilus* EF-Tus.

The G-domains from two closely related bacterial species were investigated: mesophilic *B. subtilis* (optimal growth temperature 37℃) and thermophilic *B. stearothermophilus*. The alignment of their aa sequences showed that the aa residues that differ between these two G-domains are mainly located in three distinct regions - the N-terminal, the effector, and the C-terminal region. Systematic chimerization approach and site-directed mutagenesis were used to analyze the effect of these regions on the thermostability of G-domains. The thermostabilities of chimeric/mutated G-domains were mainly assayed in functional tests – GDP or GTP binding at increasing temperature.

We demonstrated that (i) the *B. subtilis* G-domain set up the basal level of thermostability of the whole protein similarly as in the case of *E. coli* and *B. stearothermophilus* EF-Tus;

(ii) the N-terminal 12 amino acid residues played a key role in the thermostability of the G-domain. Our experiments further suggested that the thermostabilizing effect of the N-terminus is mediated by stabilizing the functionally important effector region of the G-domain;

(iii) the effect of the N-terminus was also significant for the stabilization of the full-length EF-Tu.

(Paper #4 – Šanderová et al., 2008)

4. Isolation of EF-Tus and G-domains for the analysis of polyclonal antibody against *Streptococcus pneumoniae* EF-Tu

The EF-Tus from *E. coli*, *B. stearothermophilus*, and the respective G-domains were prepared and sent to Jan Kolberg, Department of Bacteriology and Immunology, Norwegian Institute of Public Health in Oslo, Norway for experiments dealing with characterization of surface-associated EF-Tu in pneumococci and meningococci.

(Paper #5 – Kolberg et al., 2008)

4. List of Publications

1. Tomincová, H., Krásný, L., and Jonák, J. (2002). Isolation of chimaeric forms of elongation factor EF-Tu by affinity chromatography. **J. Chromatogr. B** *770*, 129-135.

2. Šanderová, H., Hůlková, M., Maloň, P., Kepková, M., and Jonák, J. (2004). Thermostability of multidomain proteins: elongation factors EF-Tu from *Escherichia coli* and *Bacillus stearothermophilus* and their chimeric forms. **Protein Sci.** *13*, 89-99.

3. Šanderová, H. and Jonák, J. (2005). Opposite roles of domains 2+3 of *Escherichia coli* EF-Tu and *Bacillus stearothermophilus* EF-Tu in the regulation of EF-Tu GTPase activity. **Biochim. Biophys. Acta** *1752*, 11-17.

4. Šanderová, H., Tišerová, H., Barvík, I., Krásný, L. and Jonák, J. (2008). N-terminal Region is Crucial for the Thermostability of the *B. stearothermophilus* G-domain. **J. Mol. Biol.** *in preparation*.

5. Kolberg, J., Hammerschmidt, S., Frank, R., Jonák, J., Šanderová, H. and Aase, A. (2008). The surface–associated elongation factor Tu is concealed for antibody binding on viable pneumococci and meningococci. **FEMS Immunology and Medical Microbiology**. *in press*.

5. Conclusions

The work presented here showed that non-catalytic domains of elongation factors Tu from mesophilic Gram negative *E. coli* and thermophilic Gram positive *B. stearothermophilus* (i) have a differentl effect on selected functions (i.e. GDP and GTP binding and GTPase activity) of EF-Tu, depending on their origin (*E. coli* or *B. stearothermophilus*), and (ii) have a similar effect on the thermostability of both EF-Tus, regardless of their origin. Furthermore, we identified thermostability elements of *B. stearothermophilus* G-domain.

This work contributes to understanding the structure-function relationship in EF-Tu proteins in general and their domain arrangement and thermostability in particular.

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