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Faculty of Science
Department of Genetics and Microbiology

EF-Tu PROTEIN DOMAINS
Functions and Thermostability

PhD thesis

Hana Šanderová

Specialization: Molecular and Cellular Biology, Genetics and Virology

Supervisor: Prof. MUDr. Jiří Jonák, DrSc.
Department of Gene Expression, Institute of Molecular Genetics AS CR

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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).
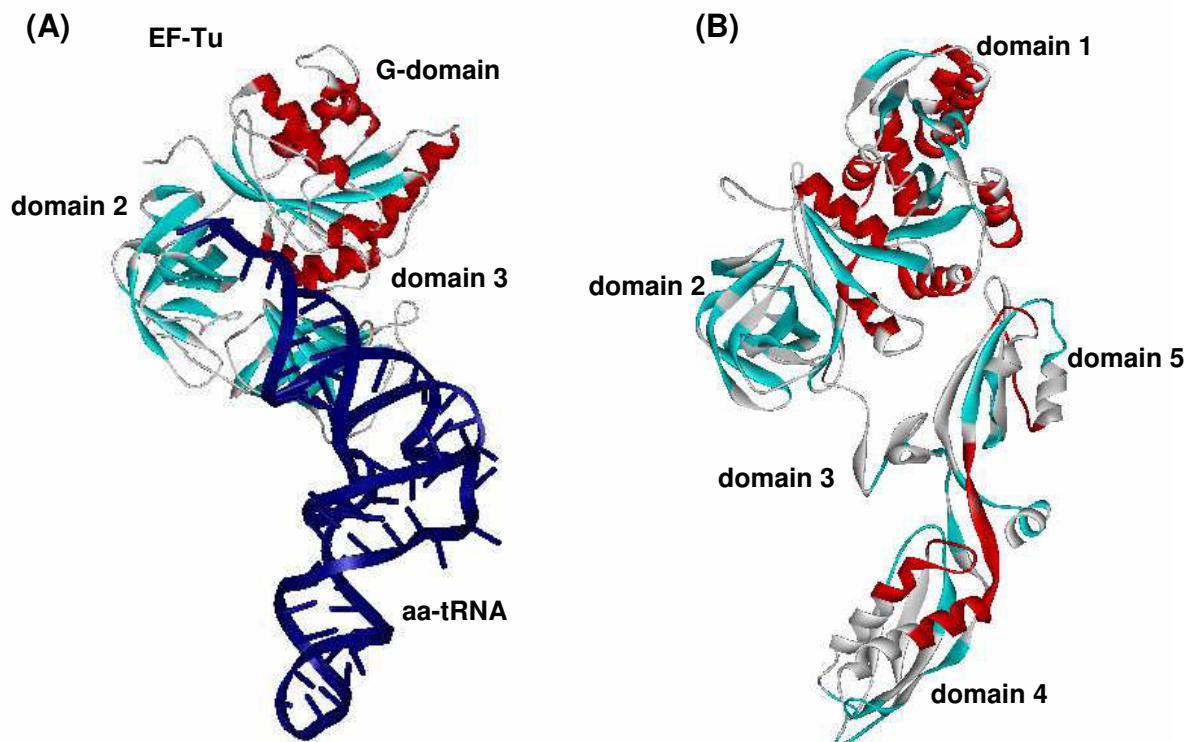

Fig. 2 Molecular mimicry between the EF-Tu·GDPNP·aa-tRNA ternary complex (A) and EF-G·GDP (B). Adopted from (Nyborg et al., 1996).

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).
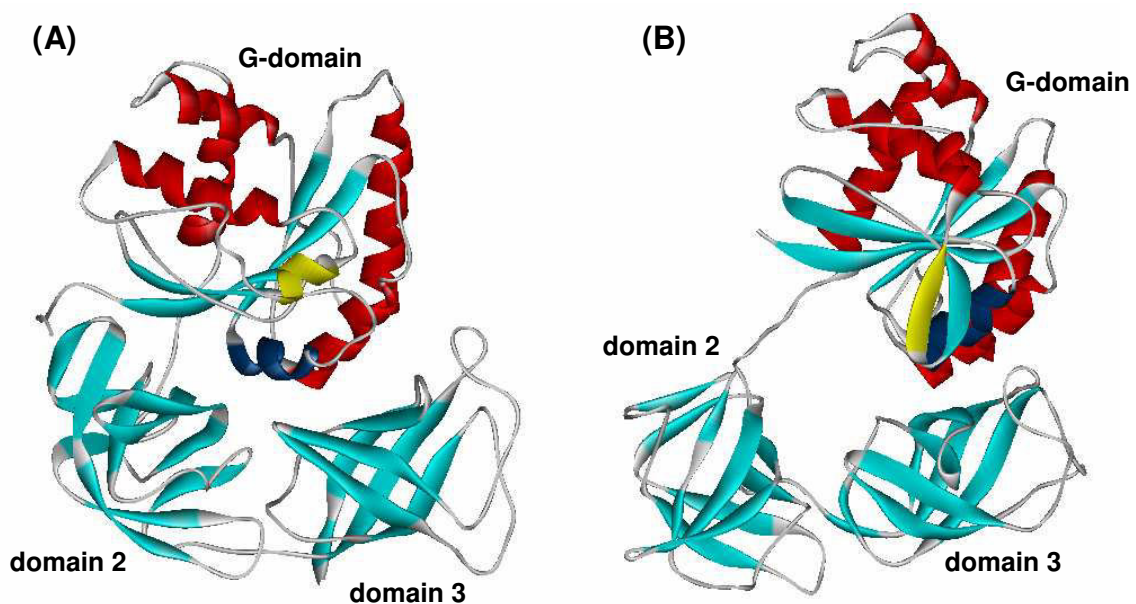

Fig. 3 3D structure of *T. aquaticus* EF-Tu in "active" GTP (A) and "inactive" GDP (B) conformation. Two parts of the G-domain that markedly change their positions and conformation after GTP hydrolysis are highlighted – the α-helix B is shown in dark blue; the α-helix A´´ that is unwound into β-strand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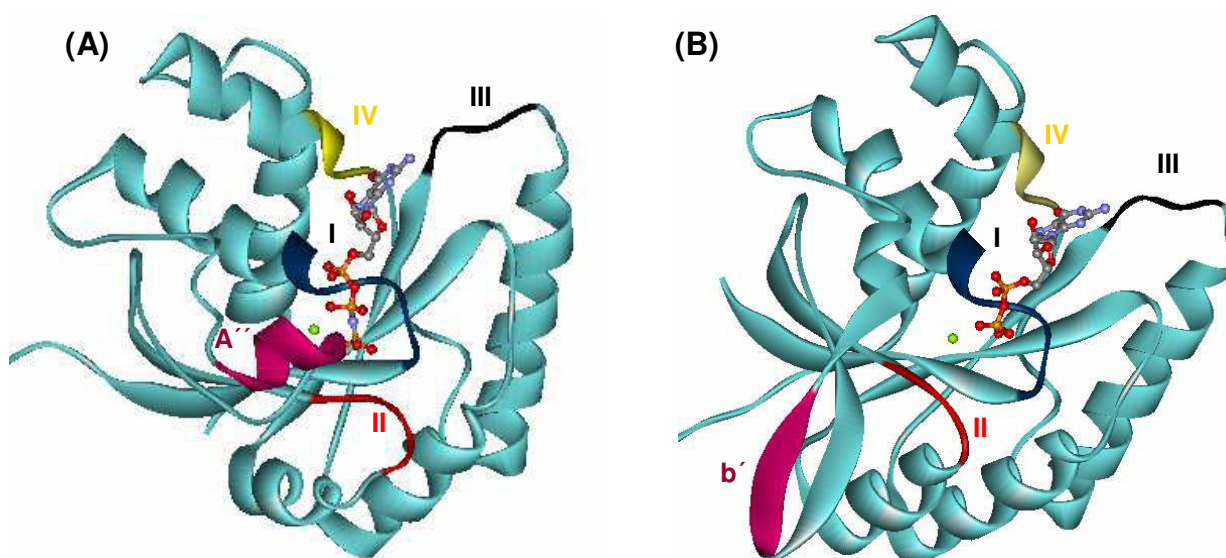

Fig. 4 3D structure of the *T. aquaticus* G-domain in the GTP (A) and GDP (B) form. The four consensus sequences are highlighted. Conserved motifs are colored (motif I in dark blue; motif II in red; motif III in black; motif IV in yellow). The unwinding of α-helix A´´ into β-list b´ triggered by GTP 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-Tu∆3 (lacking the domain 3) and EF-Tu∆2 (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-Tu∆3 and EF-Tu∆2, 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-Tu∆3 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°C m ore 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°C), in comparison with the respective EF-Tu ($\theta_{1/2}$=51°C). 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°C) 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°C). Furthermore, the domain 2 within the *T. thermophilus* EF-TuΔ3 remarkably stabilized the G-domain and this form was only by about 10°C 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°C 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°C) less thermostable than *S. solfataricus* EF-1α ($\theta_{1/2}$=96°C) and also slightly less thermostable than iso lated *S.*

solfataricus G-domain ($\theta_{1/2}$=86°C), confirming the presence of thermostabilizin g 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°C) and high (60-110°C) temperatures, a high i onic 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.
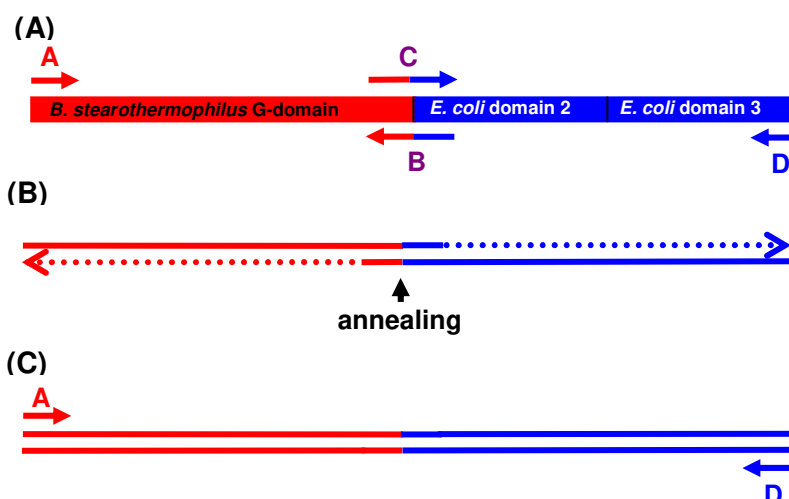

Fig. 5 Scheme of constructing a chimeric gene. (A) PCR synthesis of two fragments of a chimeric gene by primers A+B and C+D is indicated. (B) Annealing of both fragments and synthesis of whole chimeric gene. (C) Final amplification of chimeric gene by primers A+D.

  (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°C) and thermophilic *B. stearothermophilus* (optimal growth temperature 58°C) 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°C higher wi th 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°C) 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. <u>Tomincová, H.</u>, 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. <u>Šanderová, H</u>., 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. <u>Šanderová, H</u>. 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. <u>Šanderová, H.</u>, 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., <u>Šanderová, H.</u> 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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Paper 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.

Isolation of chimaeric forms of elongation factor EF-Tu by affinity chromatography

Hana Tomincová, Libor Krásný, Jiří Jonák*

Department of Protein Biosynthesis, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Flemingovo n. 2, 16637 Prague 6, Czech Republic

Abstract

Six different recombinant chimaeric forms of a three-domain protein, proteosynthetic elongation factor Tu (EF-Tu), composed of domains of EF-Tu of mesophilic (*Escherichia coli*) and thermophilic (*Bacillus stearothermophilus*) origin as well as free N-terminal domains of EF-Tu, and the whole recombinant EF-Tus of both organisms were prepared and isolated by the GST (glutathione S-transferase) fusion technology. Several modifications in the standard isolation and purification procedures are described that proved necessary to obtain the proteins in a purified and undegraded form. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Elongation factor EF-Tu; Chimaeric protein

1. Introduction

Elongation factor Tu (EF-Tu) is a protein ubiquitous in all kingdoms. It plays a central role in protein biosynthesis, where it serves in the GTP-bound form for the transport of aminoacyl-tRNA to the A-site of the mRNA-programmed ribosome. The factor also possesses a low intrinsic GTPase activity, it is a GTPase (see Ref. [1] for a review). Elongation factors Tu form a family of proteins highly homologous in primary, secondary and tertiary structure. This may be very useful for the study of evolutionary relationships between all organisms as well as for the elucidation in protein molecules of structural features of adaptation to various living conditions.

The elements of thermostability in the molecule of elongation factor Tu (EF-Tu, M_w 43,290 Da [2]) of

the moderately thermostable *B. stearothermophilus* (growth optimum 55–62°C) were investigated by creating recombinant mesophile/thermophile chimaeric forms of this three-domain protein. The chimaeric EF-Tus were composed of domains of EF-Tu from this organism combined with domains of the highly homologous (75% amino acid identity) but mesophilic EF-Tu (M_w 43,200 Da [3]) from *E. coli* (growth optimum 37°C). Although domain 1 (N-terminal or G-domain) of EF-Tu is the site of GDP/GTP binding and GTPase activity of the protein [4–6], the presence of all three domains is necessary for the binding of aminoacyl-tRNA and the function of the protein in protein biosynthesis [1]. To enable the separation of the recombinant proteins, overexpressed in *E. coli*, from the cellular *E. coli* EF-Tu, the most abundant protein in the cell, the recombinant proteins were fused with glutathione S-transferase (GST) and purified by affinity chromatography on Glutathione Sepharose 4B [7].

This approach has already proved useful for the

*Corresponding author. Tel.: +420-2-2018-3273; fax: +420-2-3333-1274.

E-mail address: jjon@img.cas.cz (J. Jonák).

preparation of many proteins and also for the preparation of EF-Tu from two organisms. *E. coli* GST–EF-Tu and its mutant forms [8], truncated forms of *E. coli* GST–EF-Tu [9] and a GST form of the wild type EF-Tu from *Bacillus subtilis* [10] were isolated by the column methods. We describe here several modifications of the standard batch isolation procedure (recommended by the manufacturer) that proved necessary to obtain 10 various recombinant forms of EF-Tu in good yield and pure and undegraded state.

2. Experimental

2.1. Chemicals and reagents

Glutathione Sepharose 4B, pGEX-5X-3 expression vector, factor Xa protease, reduced glutathione and [^{3}H]GDP (10 Ci/mmol) were purchased from Amersham Pharmacia Biotech (Prague, Czech Republic). Triton X-100 and phosphoenolpyruvate were obtained from Sigma (Prague, Czech Republic). 2-Mercaptoethanol, phenylmethyl sulphonylfluoride (PMSF), GDP (Na-salt) and GTP (Na-salt) were from Serva (Prague, Czech Republic). Isopropyl β-D-thiogalactoside (IPTG) was purchased from Amersham Pharmacia Biotech or Sigma (Prague, Czech Republic). [γ-^{32}P]GTP (5000 Ci/mmol) was provided by ICN (Zlín, Czech Republic) or Lacomed (Prague, Czech Republic) and pyruvate kinase was purchased from Calbiochem (Prague, Czech Republic). Expand High Fidelity PCR System was purchased from Roche Molecular Biochemicals (Prague, Czech Republic).

2.2. Solutions

RMK medium contained per 300 ml — 3 ml 1 *M* KCl, 6 g Bacto Tryptone, 1.5 g Bacto Yeast Extract, pH 7.6. PBS buffer (10×) was composed of 1.4 *M* NaCl, 27 m*M* KCl, 101 m*M* Na$_2$HPO$_4$, 18 m*M* KH$_2$PO$_4$, pH 7.3. Buffer A contained 1× PBS, 10 m*M* MgCl$_2$, 7 m*M* 2-ME and 15 μ*M* GDP. Buffer B (cleavage buffer) was composed of 50 m*M* Tris–Cl, pH 7.6, 100 m*M* NaCl, 10 m*M* MgCl$_2$, 1 m*M* CaCl$_2$,

7 m*M* 2-ME and 15 μ*M* GDP. Glutathione elution buffer contained 10 m*M* reduced glutathione in 50 m*M* Tris–Cl, pH 8.0, 10 m*M* MgCl$_2$ and 15 μ*M* GDP.

2.3. Methods

2.3.1. Cloning of genes for chimaeric proteins

Gene constructs encoding chimaeric EF-Tu proteins were prepared by polymerase chain reaction using Expand High Fidelity PCR System, and primary structures of recombined genes composed of defined portions of *E. coli* and *B. stearothermophilus tuf* genes (coding for EF-Tu in both organism) were verified by sequencing. The constructs were cloned into the *Bam*HI–*Eco*RI restriction site of the polylinker of the expression vector pGEX-5X-3. Recombinant proteins contained three additional amino acid residues (Gly, Ile, Pro) at the N-terminus due to the cloning into the pGEX vector polylinker and the fusion protein cleavage by factor Xa. The cloned protein genes were terminated with natural stop codons to avoid extension of encoded EF-Tu molecules at their C-end. Expression vectors with inserted gene constructs were transformed into *E. coli* strain BL21 for expression of fusion proteins.

2.3.2. Preparation of bacterial crude extract

Three hundred ml of RMK medium were supplied with 80% MgSO$_4$·5H$_2$O (10 μl/ml) and ampicillin (100 μg/ml) and inoculated with 3 ml of a night culture of *E. coli* BL21 cells transformed with pGEX vectors. Cell culture was incubated at 37°C until A_{600}=1 (Fig. 1, lane 1), then 100 m*M* IPTG was added to the final concentration 0.1 m*M* and incubation continued for 2 h (Fig. 1, lane 2). The cell culture was placed on ice and the medium was removed by centrifugation at 7700 *g*. Cells were resuspended in 15 ml of buffer A and disrupted by sonication (6×10 s with 1 min interval) at 4°C. Triton X-100 (20%) was added to the final concentration of 1% and the suspension was incubated on ice with permanent shaking for 30 min (Fig. 1, lane 3), then twice centrifuged at 12,000 *g* to remove cell debris. Supernatant was retained for the next step (Fig. 1, lane 4).
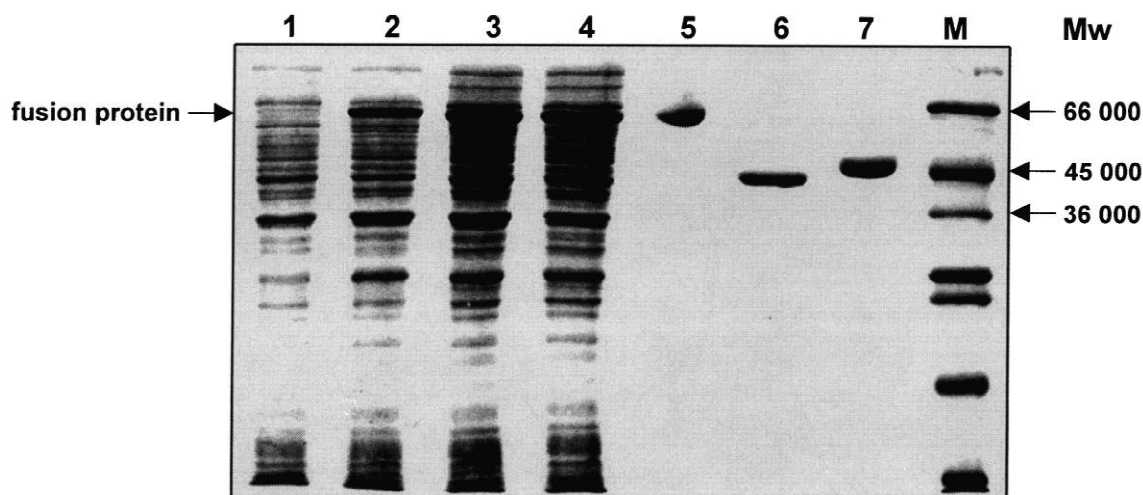
Fig. 1. Expression and purification of chimaeric EF-Tu (CH1). 12% SDS–PAGE stained with Coomassie Brilliant Blue. Non-induced cell culture (lane 1), cell culture after IPTG induction of expression of the GST–CH1 fused protein (lane 2), bacterial lysate (lane 3), supernatant for binding of the fusion protein to GS4B beads (lane 4), purified GST–CH1 protein bound to GS4B (lane 5), purified GST-free CH1 chimaeric protein (lane 6), *Bst* wtEF-Tu (lane 7). M-protein markers.

2.3.3. Preparation of 50% Glutathione Sepharose 4B

A fresh 50% (v/v, in 1× PBS) suspension of Glutathione Sepharose 4B (GS4B, agarose beads) was prepared for every experiment according to the instructions of the manufacturer.

2.3.4. Binding of GST-fused proteins to GS4B beads

Six hundred μl of 50% Glutathione Sepharose 4B (bead suspension) were added to 15 ml of the supernatant and the mixture was incubated at room temperature with permanent shaking for 30 min. The suspension was centrifuged and sedimented Glutathione Sepharose 4B beads carrying the bound fusion protein were washed four times with 15 ml buffer A and once with 15 ml buffer B (Fig. 1, lane 5).

2.3.5. Factor Xa cleavage and isolation of GST-free protein

Buffer B (450 μl) and 60 U of factor Xa (1 U/μl) were added to the Glutathione Sepharose 4B bound fusion protein suspension and the suspension was incubated at 8°C for 60 min. The cleavage reaction was stopped by addition of 1 m*M* PMSF. Agarose beads were sedimented by centrifugation and the supernatant containing the GST-free protein was transferred into a fresh tube. The sedimented beads were resuspended in 300 μl of buffer B, centrifuged and the supernatant was combined with the previous one. The combined supernatants were three times centrifuged at 10,000 *g* to remove residual agarose beads. Solutions of isolated proteins were supplemented with glycerol to the final concentration of 10% (v/v) and stored in small aliquots at −30°C. The concentration of isolated proteins was determined by the Bradford method [11] using BSA as a standard and purity was examined by SDS–PAGE (Fig. 1, lane 6).

2.3.6. Isolation of GST fusion proteins

Elution of GST fusion proteins bound to GS4B was performed in three consecutive steps each by 300 μl of the glutathione elution buffer. The suspension was incubated at 8°C, for 30 min in the first step, for 45 min in the second step and, finally, overnight. Eluted fusion protein fractions were supplemented with 10% (v/v) glycerol and stored at −30°C (Fig. 2).

2.3.7. Preparation of SDS–PAGE

The sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli [12].
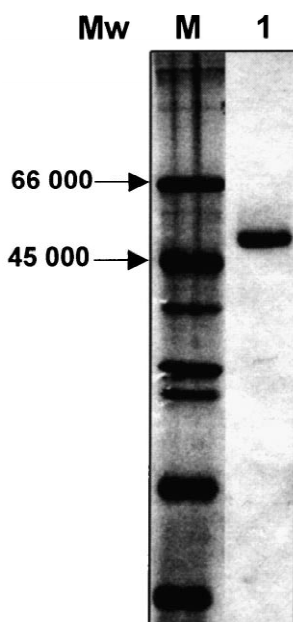
Fig. 2. 12% SDS–PAGE of GST–*Bst* G-domain fusion protein (stained with Coomassie Brilliant Blue). M-protein markers.

2.3.8. Activity of isolated proteins

The activity of proteins to bind GDP and to hydrolyze GTP in the presence of 1 *M* KCl was determined according to Anborgh et al. [13].

3. Results and discussion

Modifications of the standard GST isolation procedure introduced mainly in the GS4B binding and washing steps and in the factor Xa cleavage step that are described in the Methods and below resulted in the preparation of highly purified and undegraded recombinant products (see Fig. 3 to compare the purity of EF-Tu proteins prepared by the standard and by the modified procedure). The following recombinant EF-Tu proteins were obtained by this modified method in a pure state: recombinant *E. coli* (*Ec*) rEF-Tu, recombinant *B. stearothermophilus* (*Bst*) rEF-Tu, chimaeric EF-Tu 1 (CH1, composed of domain 1 *Ec* and domains 2+3 *Bst*), chimaeric EF-Tu 2 (CH2, composed of domains 1+3 *Ec* and domain 2 *Bst*), chimaeric EF-Tu 3 (CH3, composed of domains 1+2 *Ec* and domain 3 *Bst*), chimaeric

EF-Tu 4 (CH4, composed of domain 1 *Bst* and domains 2+3 *Ec*), chimaeric EF-Tu 5 (CH5, composed of domains 1+2 *Bst* and domain 3 *Ec*), chimaeric EF-Tu 6 (CH6, composed of domain 1+3 *Bst* and domain 2 *Ec*), isolated domain 1 (G-domain) of *E. coli* EF-Tu and isolated domain 1 (G-domain) of *B. stearothermophilus* EF-Tu (Fig. 4).

Two-hour IPTG induction was found sufficient to obtain with all constructs an ample amount of fusion proteins for isolation and purification steps by the batch method. Due to the stability requirements of the EF-Tu protein, all buffers contained 15 μM GDP and 10 mM Mg^{2+}. Recombinant proteins except the *E. coli* G-domain were obtained in a well-soluble form in *E. coli* BL21 cells. The isolation of *E. coli* G-domain will be described below.

3.1. Binding and purification of fusion proteins on GS4B beads

The binding of overexpressed EF-Tu fusion proteins from the cell extract to GS4B carried out according to the procedure recommended by the manufacturer was found to be of low efficiency; the major part of the fusion proteins remained unbound. To decrease the unbound fraction, the volume of the cell extract was reduced twice. To obtain a well-purified fusion protein, the washing steps had to be modified and various conditions had been tried. The best results were obtained by the following procedure. Firstly, the volume of the washing buffer in one washing step was increased five times and the number of washing steps was increased to five. Thorough washing after sample application was found to be critical also by Knudsen et al. [8]. Secondly and most importantly, the last washing of the beads was carried out with the cleavage buffer B to remove the still remaining fraction of non-specifically bound proteins, which would be otherwise released from the beads during the factor Xa-mediated cleavage step and contaminated the products (Fig. 3).

3.2. Cleavage of fusion proteins by factor Xa

To avoid splitting of the Arg58–Glu59 labile bond in the G-domain of *B. stearothermophilus* EF-Tu by factor Xa, the cleavage reaction took place at 8°C for
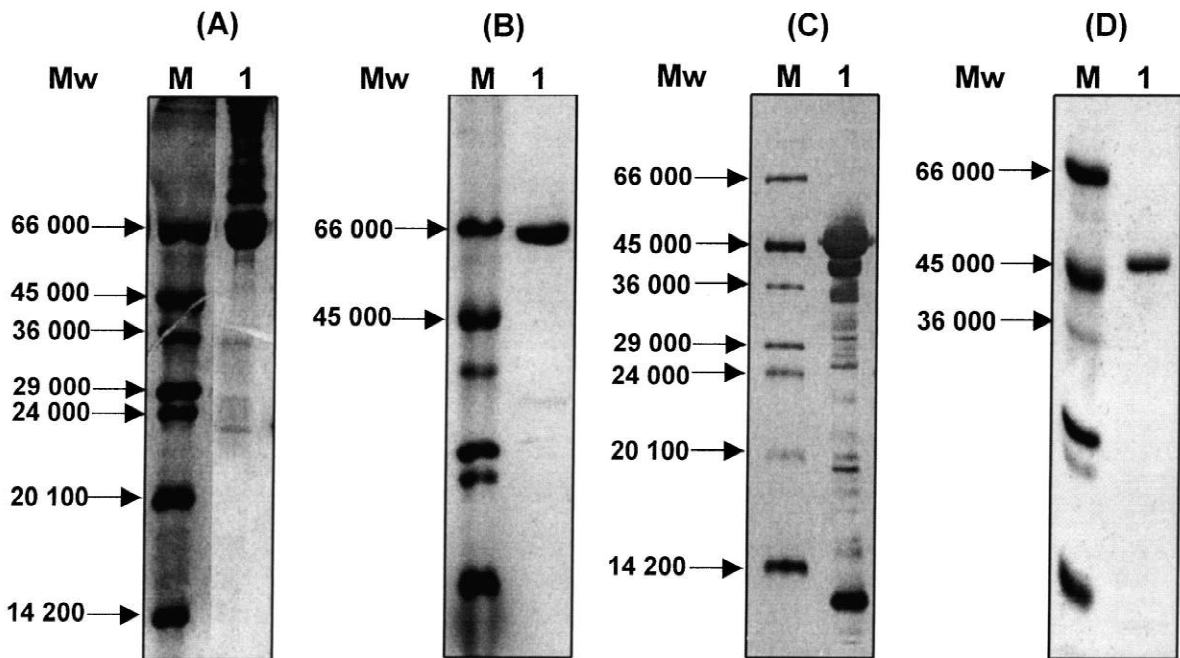
Fig. 3. Purification of EF-Tu proteins by the standard and by the modified GST-procedure: a comparison. GST–CH4 fusion protein (lane 1) purified by the standard (A) or by the modified procedure (B). GST-free recombinant EF-Tu from *B. stearothermophilus* (lane 1) purified by the standard (C) or by the modified procedure (D). M-protein markers. 15% (A, C) or 12% (B, D) SDS–PAGE stained with Coomassie Brilliant Blue (A, B, D) or with silver stain (C).

only 60 min (Fig. 5). Total inactivation of the factor Xa activity by 1 m*M* PMSF following the GST cleavage step was essential to protect the recombined EF-Tu proteins from degradation during storage. The

modifications described here were a compromise between the amount and the intactness of the isolated proteins. The average yield was between 2.3 and 4.7 μg of protein/ml of culture. The final concentration
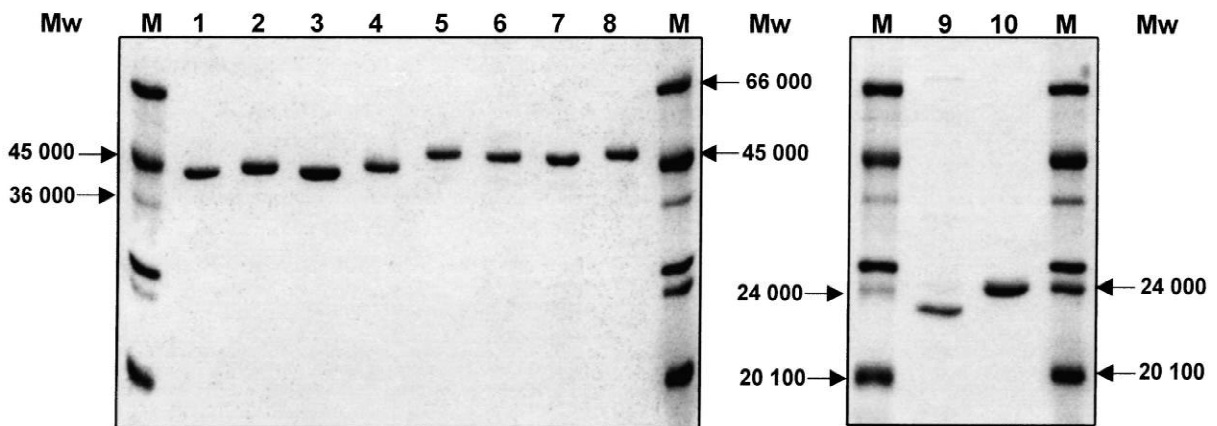

Fig. 4. 12% SDS–PAGE of isolated proteins (stained with Coomassie Brilliant Blue). *Ec* rEF-Tu (lane 1), CH1 (lane 2), CH2 (lane 3), CH3 (lane 4), *Bst* rEF-Tu (lane 5), CH4 (lane 6), CH5 (lane 7), CH6 (lane 8), *Ec* G-domain (lane 9) and *Bst* G-domain (lane 10). M-protein markers.
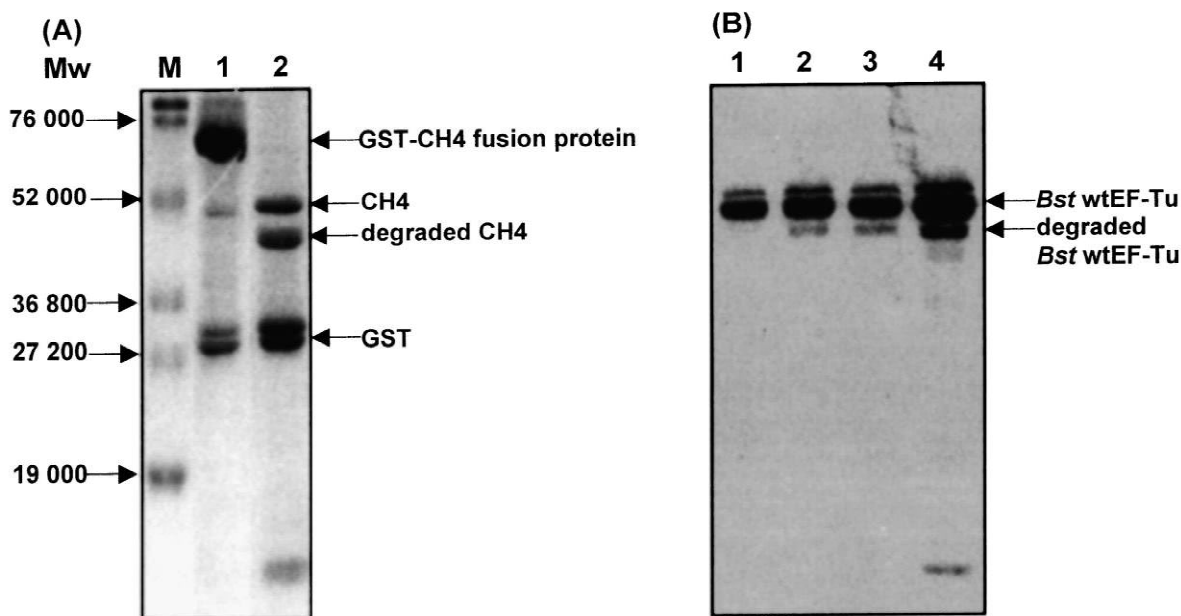
Fig. 5. Degradation of EF-Tu by prolonged factor Xa treatment. (A) Non-treated fusion protein (GST–CH4) (lane 1); fusion protein GST–CH4 treated with factor Xa at 8°C overnight (lane 2). (B) EF-Tu (*B. stearothermophilus*) treated with factor Xa at 8°C for 30 min (lane 1), 60 min (lane 2), 90 min (lane 3) and 120 min (lane 4). M-protein markers. 15% SDS–PAGE, stained with Coomassie Brilliant Blue.

of recombinant proteins obtained by the above described procedures was 0.7–1.6 μg/μl.

3.3. Elution of fusion proteins

The elution procedure for GST-fused EF-Tu proteins from GS4B beads was also modified. The elution with the glutathione elution buffer was carried out at 8°C instead of at room temperature and the elution times were increased to 30, 45 min and overnight incubation (instead of 10 min). The longer elution time, the less fusion protein remained bound to GS4B. The average yield of the GST-fused EF-Tu proteins was about 16 μg of protein/ml of culture and the concentration was 3.1–7.8 μg/μl in individual elution steps.

3.4. Preparation of the E. coli G-domain of EF-Tu

A major part of the GST–G-domain fusion protein was obtained in an insoluble form and only a minor part of the protein stayed soluble, even though, fortunately, both the recombinant *E. coli* GST–EF-Tu and the chimaeric GST-forms of EF-Tu with the *E. coli* G-domain were all soluble. Similarly, Parmeggiani et al. [5] also reported that the non-fused *E. coli* EF-Tu G-domain overexpressed in *E. coli* cells was mostly insoluble. The protocol for the isolation of sufficient amount of the *E. coli* EF-Tu G-domain was modified in the following way: 900 ml of the bacterial culture were incubated at 28–29°C until $A_{600}=0.8$, then expression of the GST–G-domain fusion protein was induced by 0.1 mM IPTG and the incubation continued for 2 h. The insoluble form of the GST–G-domain protein was removed from the bacterial crude extract by centrifugation and only the soluble portion of the fusion protein was used for further purification by affinity chromatography on GS4B and factor Xa cleavage. The beads were washed four times with buffer A and three times with buffer B. The yield of the soluble and purified *E. coli* G-domain was 0.2–0.3 μg of protein/ml of culture in the concentration of 0.8 μg/μl.

Table 1
Activity of isolated recombinant EF-Tu proteins

Protein	GDP binding (mol/mol)	Optimal temperatures of the GTPase activity (°C)
Ec rEF-Tu	0.4	37
CH1 ($G_{Ec}-2+3_{Bst}$)	0.34	48
CH2 ($G_{Ec}-2_{Bst}-3_{Ec}$)	0.3	45
CH3 ($G+2_{Ec}-3_{Bst}$)	0.45	44
Ec G-domain	0.05	35
Bst rEF-Tu	0.35	61
CH4 ($G_{Bst}-2+3_{Ec}$)	0.3	51
CH5 ($G+2_{Bst}-3_{Ec}$)	0.31	55
CH6 ($G_{Bst}-2_{Ec}-3_{Bst}$)	0.34	58
Bst G-domain	0.42	55

3.5. Activity of recombinant proteins

As shown in Table 1, six chimaeric forms of EF-Tu, representing all possible combinations of protein domains of EF-Tu from *E. coli* and *B. stearothermophilus*, as well as recombinant EF-Tus and free recombinant G-domains of EF-Tu of both organisms prepared by the modified GST method were found to be active in GDP binding and to efficiently hydrolyze GTP. Whereas the free *Bst* G-domain possesses GDP-binding activity comparable with that of all three-domain-forms of EF-Tu, the free *Ec* G-domain is much less active. Low binding activity of the *Ec* G-domain prepared by a different procedure was also observed by Parmeggiani et al. [5]. GTPase activity of all proteins was examined as a function of increasing temperature to determine the relationship between the structure of the proteins and their thermophilicity. The results show that the temperature optimum for the GTPase activity of each protein is primarily dependent on the origin of the G-domain but the origin of the other two domains, domain 2 and domain 3, may have a significant modulatory effect. Substitution of domains 2 and 3 of the thermophilic *Bst* EF-Tu by domains 2 and 3 from *Ec* EF-Tu (chimaeric EF-Tu protein CH4) results in about 10°C decrease in the GTPase activity temperature optimum as compared to the intact *Bst* EF-Tu whereas the opposite substitution represented

by chimaeric EF-Tu protein CH1 results in about 10°C increase in the temperature optimum, as compared to *Ec* EF-Tu. These results complemented with determination of thermostability of all individual protein products measured both in functional assays [14] and by physical methods will help to elucidate mechanisms of thermostabilization in this three-domain protein functioning in all prokaryotes from halophiles to hyperthermophiles.

Acknowledgements

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Paper 2

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Thermostability of multidomain proteins: Elongation factors EF-Tu from *Escherichia coli* and *Bacillus stearothermophilus* and their chimeric forms

HANA ŠANDEROVÁ,[1] MARTA HŮLKOVÁ,[1] PETR MALOŇ,[1,2]
MARKÉTA KEPKOVÁ,[1] AND JIŘÍ JONÁK[1]

[1]Department of Protein Biosynthesis, Institute of Molecular Genetics and [2]Department of Peptide Chemistry,
Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic,
Prague 6, Czech Republic

Abstract

Recombinant mesophilic *Escherichia coli* (Ec) and thermophilic *Bacillus stearothermophilus* (Bst) elongation factors EF-Tus, their isolated G-domains, and six chimeric EF-Tus composed of domains of either EF-Tu were prepared, and their GDP/GTP binding activities and thermostability were characterized. BstEF-Tu and BstG-domain bound GDP and GTP with affinities in nanomolar and submicromolar ranges, respectively, fully comparable with those of EcEF-Tu. In contrast, the EcG-domain bound the nucleotides with much lower, micromolar affinities. The exchange of domains 2 and 3 had essentially no effect on the GDP-binding activity; all complexes of chimeric EF-Tus with GDP retained K_d values in the nanomolar range. 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 "basic" level of the thermostability, which was ~20°C higher with the BstG-domain than with the EcG-domain. This correlated with the growth temperature optimum difference of both bacteria and two distinct thermostabilization features of the BstG-domain: 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 and, in turn, the functions of the G-domains to the level of the respective growth temperature optima. Their contributions were similar irrespective of their origin but, with Ecdomains 2 + 3, dependent on the guanine nucleotide binding state. It was lower in the GTP conformation, and the mechanism involved the destabilization of the α-helical regions of the G-domain by Ecdomain 2.

Keywords: EF-Tu; thermostability; chimeric protein; EF-Tu domains; G-domain; *Escherichia coli*; *Bacillus stearothermophilus*

Elongation factors EF-Tu/EF-1α are abundant, highly homologous cellular GTP-proteins occupying a key position in translation in all organisms as universal carriers of aminoacyl-tRNAs. Their conformation and activity are regulated by GDP and GTP (Jonák and Rychlík 1973; Printz and Miller 1973; Kaziro 1978), and they hydrolyze bound GTP (Krab and Parmeggiani 1998). Their known 3D structures are superimposable, and they share the same catalytic mechanisms (Krab and Parmeggiani 1998). The high structural homology predetermines the elongation factors for the study of evolutionary relationships between organisms (Baldauf et al. 1996) and for elucidation of the structural features of adaptation to various living conditions.

All EF-Tus/EF-1αs are monomeric proteins composed of ~400 amino acid residues (for review, see Krab and Parmeggiani 1998) folded into three clearly distinct domains (Kjeldgaard and Nyborg 1992; Berchtold et al. 1993; Song et al. 1999).

N-terminal domain 1 (~200 residues) of EF-Tu is composed of a predominantly parallel six-stranded β-sheet core surrounded by seven α-helices. It contains the guanine nucleotide-binding site and the GTPase center (Parmeggiani et al. 1987; Kjeldgaard and Nyborg 1992) and is called the catalytic or G-domain (Parmeggiani et al. 1987). Middle domain 2 (~100 residues) and C-terminal domain 3 (~100 residues) are β-barrels of seven and six antiparallel β-strands, respectively, that share an extended interface. The reasons for the arrangement of EF-Tu into three domains are not fully understood except for the fact that formation of the aminoacyl-tRNA-binding site requires participation of all of them. This takes place in the GTP conformation, in which EF-Tus exist in a compact form with tight interfaces between the G-domain and domain 2 as well as the G-domain and domain 3 and which has a high affinity for aminoacyl-tRNAs and ribosomes (Berchtold et al. 1993; Kjeldgaard et al. 1993). Hydrolysis of the bound GTP during the mRNA decoding induces a large conformational change characterized by an opening of the molecule. The interactions between the G-domain and domain 2 are essentially lost, and only interactions between domain 3 and domain 2 are preserved while new interactions are formed between domain 3 and the G-domain. EF-Tu in the open-GDP conformation has a low affinity for aminoacyl-tRNA (Jonák et al. 1980).

Until now, functional evaluation of individual domains of EF-Tu has been carried out mainly by the deletion approach, by examining one- or two-domain proteins (Parmeggiani et al. 1987; Nock et al. 1995; Masullo et al. 1997; Cetin et al. 1998). In this paper, we address the question of how the functions and thermostability of EF-Tu from mesophilic *Escherichia coli*, growing at ~37°C and EF-Tu from thermophilic *Bacillus stearothermophilus* growing at temperatures 55°–60°C are built from the contributions of individual domains. Identification of the molecular basis of the increased thermostability of the proteins is expected to help our understanding of protein folding as well as the design of enzymes retaining their activity at high temperature. First, molecules of the EF-Tus were genetically dissected into three corresponding domains, and the domains were combined to form six chimeric EF-Tu proteins. In addition, the G-domains, the functional modules of EF-Tus of both organisms, were prepared and examined. Second, the GDP- and GTP-binding activities and thermostability of the proteins were measured both as the maintenance, at increasing temperatures, of a defined functional state (Jaenicke and Böhm 1998) by the ability to bind GDP and GTP and, independently, using CD spectroscopy, as the maintenance of the α-helix content. There were several reasons for the application of the chimerization approach: (1) The proteins share 75% amino acid sequence identity (Krásný et al. 1998); (2) *B. stearothermophilus* EF-Tu (M_r = 43,290 D, 394 amino acid residues) is only one amino acid residue

longer than *E. coli* EF-Tu (M_r = 43,200 D, 393 amino acid residues; Jones et al. 1980; Krásný et al. 1998); (3) *E. coli* and *B. stearothermophilus* elongation factors and ribosomes are functionally interchangeable (Jonák et al. 1986 and references therein); and (4) the properties of the domains could be evaluated within full-length, three-domain proteins. Finally, we focused our attention on structural features that could lead to different thermal stabilities of both EF-Tus. Our results indicate that their thermostability is the result of cooperative interaction between the G-domains and domains 2 + 3 and provide insight into why the EF-Tu from *B. stearothermophilus* is more thermostable than the EF-Tu from *E. coli*.

Results

Recombinant EF-Tu proteins

Ten proteins were prepared by the GST-purification technology: recombinant *E. coli* EF-Tu (EcEF-Tu); recombinant *B. stearothermophilus* EF-Tu (BstEF-Tu); chimera 1 (CH1, composed of Ecdomains 1 + 3 and Bstdomain 2); chimera 2 (CH2, composed of Ecdomains 1 + 2 and Bstdomain 3); chimera 3 (CH3, composed of Ecdomain 1 and Bstdomains 2 + 3); chimera 4 (CH4, composed of Bstdomains 1 + 3 and Ecdomain 2); chimera 5 (CH5, composed of Bstdomains 1 + 2 and Ecdomain 3); chimera 6 (CH6, composed of Bstdomain 1 and Ecdomains 2 + 3); the EcG-domain; and the BstG-domain. Each protein moved as a single band on SDS-electrophoresis gels (Fig. 1). Except for the EcG-domain, the proteins were 70%–100% active in binding GDP in the 1 : 1 molar ratio. EcG-domain preparations were only 7%–12% active irrespective of the two different methods of preparation described in Materials and Methods. All the data presented below apply to 100% active proteins.

EcEF-Tu, BstEF-Tu, and their chimeric variants differed in their electrophoretic mobility in 12% SDS-polyacrylamide gel (Fig. 1) even though, according to the amino acid composition, their M_rs are essentially identical. BstEF-Tu
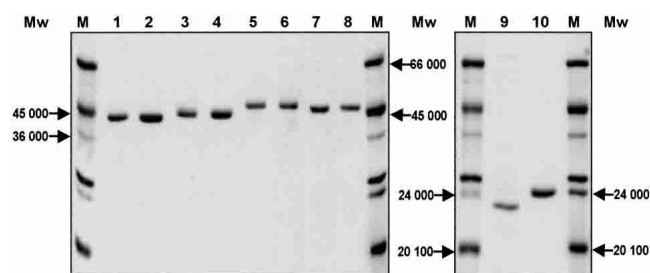

Figure 1. 12% SDS-PAGE of isolated proteins (stained with Coomassie brilliant blue). EcEF-Tu (lane *1*), CH1 (lane *2*), CH2 (lane *3*), CH3 (lane *4*), BstEF-Tu (lane *5*), CH4 (lane *6*), CH5 (lane *7*), CH6 (lane *8*), EcG-domain (lane *9*), and BstG-domain (lane *10*). (M) Molecular weight protein markers.

moved considerably more slowly than EcEF-Tu, indicating the M_r of BstEF-Tu to be ~7 kD higher than that of EcEF-Tu (see also Wittinghofer and Leberman 1976; Wormer et al. 1983; Jonák et al. 1986). Our experiments show that the mobility difference between EcEF-Tu and BstEF-Tu can already be traced down to their G-domains: (1) The isolated BstG-domain was markedly slower than the isolated EcG-domain even though the G-domains did not essentially differ in M_r or pI (data not shown); (2) chimeric EF-Tus comprising the BstG-domain were slower than those with the EcG-domain. Exchange of domains 3 but not domains 2 further modulated the mobility.

Interaction of EF-Tus, chimeric forms of elongation factors Tu, and isolated G-domains with GDP and GTP

The parameters governing the interaction between the proteins and GDP or GTP are summarized in Table 1. The K_d of the BstEF-Tu · GDP complex lay in the nanomolar range (4.17 nM) and the K_d of the BstEF-Tu · GTP complex in the submicromolar range (295 nM). They closely correlated with the K_ds of the analogous complexes of EF-Tu from *E. coli* and so did the association and dissociation rates of the complexes of both organisms.

The kinetic parameters of the GDP complexes of chimeric EF-Tus indicate that the exchange of domains did not compromise the GDP-binding ability of the six new artificial proteins, chimeras CH1–CH6, as compared with EF-Tu of *E. coli* or *B. stearothermophilus* (Table 1). All chimeric EF-Tus were found to bind GDP with high affinity, with K_ds in the nanomolar range (0.8–3.5 nM).

On the other hand, the kinetic parameters of the GDP and GTP complexes of the isolated EcG-domain, in particular, k_{+1} and K_d, strongly differed from those of the BstG-domain

(Table 1). The BstG-domain bound GDP and GTP with the affinity almost as strong as that of BstEF-Tu. In contrast, the affinity of the EcG-domain for GDP and GTP was about three orders of magnitude and more than one order of magnitude lower, respectively, than those of EcEF-Tu (Table 1; Fasano et al. 1978; Jensen et al. 1989; Cetin et al. 1998). The difference in the affinity for GDP or GTP between the BstG-domain and the EcG-domain was mainly caused by the fact that the k_{+1}s of the BstG-domain · GDP/GTP complexes were more than one to two orders of magnitude greater than those of the EcG-domain · GDP/GTP complexes.

Heat stability of GDP forms of the G-domains, EF-Tus, and their variants

The heat inactivation profiles of all individual proteins were determined and their $\theta_{1/2}$s, the temperatures at which half of their maximal GDP binding activity was lost, are summarized in Table 2. According to the $\theta_{1/2}$ values, the BstG-domain ($\theta_{1/2}$ = 45.5°C) was ~20°C more thermostable than the EcG-domain ($\theta_{1/2}$ = 25.8°C) and ~18°C less thermostable than BstEF-Tu ($\theta_{1/2}$ = 63.8°C). The difference in $\theta_{1/2}$ between EcG-domain · GDP and EF-Tu · GDP ($\theta_{1/2}$ = 49.1°C) was ~23°C. With respect to $\theta_{1/2}$, the mesophilic EcEF-Tu in the GDP conformation was ~15°C less thermostable than the thermophilic BstEF-Tu · GDP (Fig. 2). The exchange of one noncatalytic domain of BstEF-Tu for the homologous Ecdomain, as in CH4 and CH5, was already sufficient to decrease the thermostability of its GDP form. In contrast, the substitution of one noncatalytic domain of EcEF-Tu · GDP for the homologous Bstdomain, as in CH1 and CH2, was not sufficient, contrary to expectation, to increase it, but actually decreased it. Only the ex-

Table 1. *Kinetic parameters of interaction of* E. coli *and* B. stearothermophilus *EF-Tus, G-domains, and CH1–CH6 chimeric EF-Tus with GDP and GTP*

Protein	GDP (0°C)			GTP (0°C)		
	k_{-1} ($\times 10^4$ sec^{-1})	k_1 ($\times 10^4$ M^{-1} sec^{-1})	K_d (nM)	k_{-1} ($\times 10^4$ sec^{-1})	k_1 ($\times 10^{-4}$ M^{-1} sec^{-1})	K_d (nM)
Ec G-domain	23.73	0.13	*1825.38*	58–115	*0.09–0.18*	*5100–8000*
EC EF-Tu	6.58	14.20	*4.63*	141.11	5.06	309.15
CH1	7.37	43.12	*1.71*	n.d.	n.d.	n.d.
CH2	2.37	29.94	*0.79*	n.d.	n.d.	n.d.
CH3	6.97	37.43	*1.86*	n.d.	n.d.	n.d.
Bst G-domain	16.44	40.16	6.25	121.38	5.55	218.58
Bst EF-Tu	6.90	21.56	4.17	94.21	3.20	294.73
CH4	3.42	17.98	*1.90*	n.d.	n.d.	n.d.
CH5	6.25	17.94	*3.48*	n.d.	n.d.	n.d.
CH6	2.14	26.22	*0.82*	n.d.	n.d.	n.d.

Reaction conditions are described in Materials and Methods. All results were obtained in several independent assays, and the values are the averages. Values in italics were calculated using $k_1 = k_{-1}/K_d$. Values of k_1, k_{-1}, and K_d for the *E. coli* G-domain · GTP complex were obtained from the references of Parmeggiani et al. (1987), Cetin et al. (1998), and Jensen et al. (1989). (n.d.) Not determined.

Table 2. *Parameters characterizing the thermal stability of* E. coli *and* B. stearothermophilus *EF-Tus, G-domains, and CH1–CH6 chimeric EF-Tus*

Protein	$\theta_{1/2}$ (GDP) (°C)	$\theta_{1/2}$ (GTP) (°C)	T_m CD (GDP) (°C)	T_m CD (GTP) (°C)
Ec G-domain	25.8	29	n.d.	n.d.
Ec EF-Tu	49.1	41.2	46.5	42.2
CH1 (G$_{Ec}$ − 2$_{Bst}$-3$_{Ec}$)	47.8	42.6	46.2	n.d.
CH2 (G 2$_{Ec}$ − 3$_{Bst}$)	47.8	39	45.5	n.d.
CH3 (G$_{Ec}$-2 3$_{bst}$)	51.8	51.6	46.9	47
Bst G-domain	45.5	47.3	42	44
Bst EF-Tu	63.8	63	57.5	58
CH4 (G$_{Bst}$ − 2$_{Ec}$ − 3$_{Bst}$)	62	58.2	57.1	n.d.
CH5 (G 2$_{Bst}$ − 3$_{Ec}$)	55.2	55.2	54	n.d.
CH6 (G$_{Bst}$-2 3$_{Ec}$)	59	53.8	55.5	50.8

Reaction conditions are described in Materials and Methods. $\theta_{1/2}$ is the temperature of half-inactivation of the protein in GDP or GTP binding. T_m is the temperature of 50% change in the α-helix content of the protein in the GDP or GTP conformation. (n.d.) Not determined. All values are the means from at least three independent experiments.

change of both noncatalytic domains 2 + 3 for the homologous Bstdomains, as in CH3, resulted in a slight enhancement (2.7°C) of the thermostability as compared with EcEF-Tu · GDP.

The thermostability profiles of the wild-type EF-Tus isolated from *E. coli* or *B. stearothermophilus* were essentially identical to the profiles of the recombinant EF-Tus (data not shown).

Heat stability of GTP forms of the G-domains, EF-Tus, and their variants

Conversion of the proteins from GDP to GTP conformation differently affected their heat stability. The $\theta_{1/2}$ of EcEF-Tu

Figure 2. Heat inactivation profiles of GDP (closed symbols) and GTP (open symbols) forms of *E. coli* and *B. stearothermophilus* EF-Tus and G-domains. *Ec*EF-Tu (gray ▲), *Ec*G-domain (gray ◆), *Bst*EF-Tu (black ▲), and *Bst*G-domain (black ◆).

decreased by 8°C from 49.1°C to 41.2°C, whereas the $\theta_{1/2}$ of BstEF-Tu remained essentially unchanged. In contrast, both the EcG-domain and BstG-domain displayed a higher thermostability in the GTP conformation than in the GDP conformation (Fig. 2; Table 2). The replacement in EcEF-Tu of domains 2 + 3 by Bstdomains 2 + 3, as in CH3, protected the protein from the decrease in thermostability of its GTP form. Values of $\theta_{1/2}$ equal to 51.8°C for CH3 · GDP and 51.6°C for CH3 · GTP were obtained (Table 2). The replacement in BstEF-Tu of domains 2 + 3 by Ecdomains 2 + 3, as in CH6, had an opposite effect, rendering its thermostability sensitive to the type of the bound guanine nucleotide, similarly as in EcEF-Tu. The $\theta_{1/2}$ of CH6 · GTP was more than 5°C lower than the $\theta_{1/2}$ of CH6 · GDP (Table 2). The swapping of only one domain provided a pattern of results similar to that obtained with the proteins in the GDP conformation.

Unfolding of the G-domains, EF-Tus, and their variants by heat

To compare the functional thermostability profiles of individual proteins and their structural stability, preservation of the α-helix content of the proteins at increasing temperature was measured by CD spectroscopy.

As the first result, each product showed a CD spectrum of a protein with a defined structure. The CD spectra of chimeric EF-Tus matched those of BstEF-Tu or EcEF-Tu (data not shown), implying that the organization of the secondary structural elements in the chimeras was not dramatically changed. The negative CD at 220 nm, indicative of the presence of α-helices, was larger in G-domains than in the three-domain EF-Tu variants (Fig. 3). This difference is to be expected because the deletion of the middle and C-terminal domain removes parts of EF-Tu that contain only β-strands. Such secondary structure composition of domains 2 and 3 revealed by X-ray diffraction analysis for EcEF-Tu (Kjeldgaard and Nyborg 1992; Berchtold et al. 1993; Song et al. 1999) was also proposed to hold for domains 2 and 3 of BstEF-Tu (Krásný et al. 1998).

The CD spectrum of each protein displayed a characteristic temperature-induced transition change. The change (in percent) in the helicity of the proteins in GDP or GTP conformation detected at 220 nm was plotted against the temperature. The mid-temperatures (T_m) of the transition from the α-helical conformation to disordered conformation (unfolding) determined for every protein are summarized in Table 2. The T_m of the isolated BstG-domain · GDP was 42°C. Its conversion into the GTP conformation increased the T_m by 2°C. The CD spectrum of the EcG-domain could not be considered as representative because the concentration of the protein in an active conformation was low (see above). The T_ms of GDP forms of the three-domain proteins comprising the BstG-domain (BstEF-Tu, chimeras CH4–
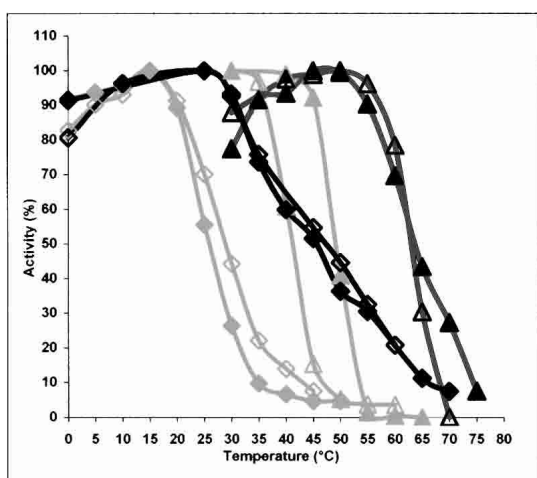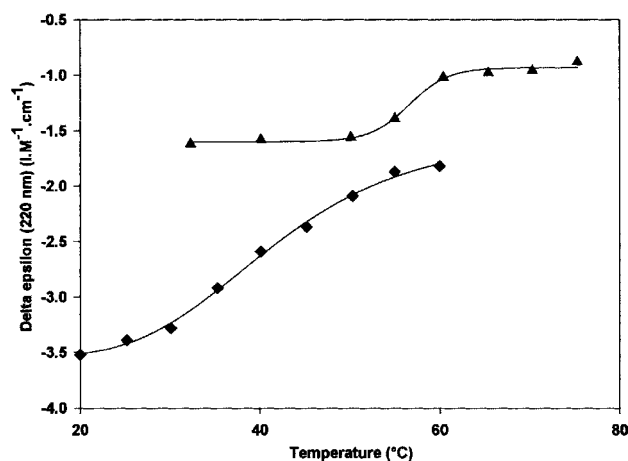
Figure 3. The change in CD of BstEF-Tu (▲) and BstG-domain (♦) measured at 220 nm as a function of increasing temperature.

CH6) were 12°–15.5°C higher than the T_m of the BstG-domain alone. They lay in the range of 54°–57.5°C. The T_ms of the GDP forms of EcEF-Tu and chimeras CH1–CH3, comprising the EcG-domain, lay in the range of 45.5°–46.9°C (Table 2). The mid-temperature of α-helix unfolding of EcEF-Tu decreased by 4.3°C, to 42.2°C, when the protein in the GTP conformation was assayed. On the other hand, upon conversion of BstEF-Tu from the GDP to GTP conformation, an ~0.5°C increase in the T_m was observed (Fig. 4; Table 2). Swapping of domains 2 + 3 between both EF-Tus influenced sensitivity of the unfolding to the type of bound guanine nucleotide analogously as in the case of the functional thermostability. The T_m displayed by CH6 · GTP, composed of the BstG-domain and Ecdomains 2 + 3, was ~5°C lower than the T_m of CH6 · GDP. On the other hand, the T_m of CH3, consisting of the EcG-domain and Bst domains 2 + 3, was not influenced by bound guanine nucleotide. The thermal stability of α-helices in CH3 · GDP and CH3 · GTP was essentially identical (Table 2). The temperature-induced change in the helicity of the three-domain proteins was typically completed within a temperature range of ~15°C. However, an ~30°C temperature range was required to complete the denaturation of α-helices of the separated BstG-domain (Fig. 3). The figure also shows that the α-helix denaturation started in the isolated BstG-domain at a lower temperature than in the three-domain proteins.

Discussion

Domains of EcEF-Tu and BstEF-Tu and guanine nucleotide-binding activity

All 10 recombinant proteins were active in the binding of GDP and GTP and GTP hydrolysis (data not shown). The affinity characteristics of the thermophilic BstEF-Tu for GDP and GTP, determined here for the first time, closely matched those of the mesophilic EcEF-Tu (Table 1; Arai et al. 1974; Miller and Weissbach 1977; Fasano et al. 1978; Sanangelantoni et al. 1996; Cetin et al. 1998). GDP was bound by either factor about 100 times more strongly than GTP, mainly as the consequence of about 10–20 times greater k_{-1} of EF-Tu · GTP complexes. This behavior has been considered typical of bacterial EF-Tus. As it is shown here, it holds for BstEF-Tu as well, although GTP, owing to the extra phosphate residue, has additional interactions with the proteins as compared with GDP (Berchtold et al. 1993; Kjeldgaard et al. 1993). No explanation in terms of the 3D structure for the different affinity of EF-Tus for GDP and GTP has been found as yet.

Comparison of the GDP- and GTP-binding properties of isolated G-domains provided different results. The BstG-domain possessed affinities for GDP and for GTP quite comparable with those of the intact BstEF-Tu. In contrast, the removal of domains 2 + 3 from EcEF-Tu resulted in a profound loss in the binding affinity for guanine nucleotides, particularly for GDP. The K_d increased almost 1000 times from the nanomolar range to the micromolar range (Table 1; Parmeggiani et al. 1987; Cetin et al. 1998). As reported from other laboratories, the affinity of the EcG-domain for GTP also decreased as compared with that of EcEF-Tu · GTP, but only about 20 times (Parmeggiani et al. 1987; Jensen et al. 1989; Cetin et al. 1998), so that the binding of GDP and GTP by the isolated EcG-domain took place with almost the same (low) affinity (Table 1).

These data imply that whereas in BstEF-Tu, the high and different affinity for GDP and GTP appears to be intrinsic to the G-domain itself, in *E. coli*, the cooperation involving all three domains of EF-Tu is required to establish this phenotype (cf. also Cetin et al. 1998). Nock et al. (1995) reported
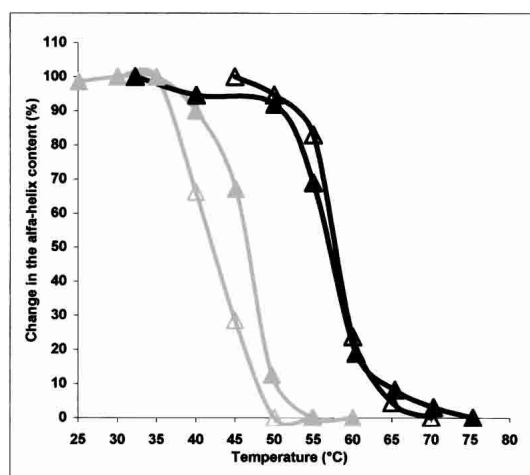

Figure 4. Temperature-dependent change in the α-helix content of GDP (closed symbols) and GTP (open symbols) forms of *E. coli* EF-Tu (gray lines) and *B. stearothermophilus* EF-Tu (black lines).

that in *Thermus thermophilus* EF-Tu, the deletion of domains 2/3 or only of domain 3 provoked a lower affinity for guanine nucleotides similarly as in the *E. coli* system. In contrast, the same truncation of *Sulfolobus solfataricus* EF-1α increased the affinity for GDP and GTP by about one order of magnitude compared with the intact protein (Masullo et al. 1997). This implies that the same level of affinity to guanine nucleotides can be attained by different strategies in different EF-Tus.

We did not check whether the separated G-domains mixed with domains 2/3 could form any functional complexes. It was demonstrated that a mixture of the G-domain and domains 2/3 of *T. thermophilus* EF-Tu could neither form any detectable complexes nor function in poly(U)-dependent poly(Phe) synthesis (Nock et al. 1995). This indicated that proper covalent linking of the three domains of EF-Tu is required for the formation of specific interdomain interactions found in the crystal structure of the protein and, in turn, for its functioning.

Thermostability of EF-Tu: Domain cooperation

Our studies with EcEF-Tu and BstEF-Tu and their domains indicate that the final level of thermostability of the three-domain EF-Tus is attained by a cooperative interaction between G-domains and domains 2 + 3. The G-domains set up a "basic level" of the thermal stability of the EF-Tus (cf. also Sanangelantoni et al. 1996; Masullo et al. 1997). The level set up by the thermophilic BstG-domain was ~20°C higher than that of the EcG-domain. In turn, this difference in $\theta_{1/2}$ between the G-domains fully correlated with the difference in $\theta_{1/2}$ between intact EF-Tus. In absolute terms, the $\theta_{1/2}$ of the G-domains were still ~20°C below those of the respective EF-Tus.

Both the EcG-domain and the BstG-domain were a little more thermostable in the GTP conformation than in the GDP conformation, which may be because of the extra interaction with the γ-phosphate residue of GTP (Kjeldgaard et al. 1993).

The domains 2 + 3 were found to enhance the thermal stability of the α-helical structure of the G-domain to the level consistent with the bacterial growth temperature optimum. The unfolding of the α-helical regions of the G-domain within the three-domain EF-Tu started at higher temperatures than in the G-domain alone (Fig. 3). In contrast to the G-domains, the gain in thermostability due to domains 2 + 3 indicated by $\theta_{1/2}$ was (1) similar, irrespective of the domain origin, and (2) smaller in the GTP complexes than in the GDP complexes. The conversion to the GTP conformation decreased particularly the $\theta_{1/2}$ and T_m of those proteins comprising Ecdomains 2 + 3 as was the case for EcEF-Tu and chimera CH6, composed of the BstG-domain and Ecdomains 2 + 3. The physiological consequences of the different stability of the GDP and GTP forms of *E. coli*

EF-Tu have not been understood. Comparison of $\theta_{1/2}$ of GDP and GTP forms of the tested proteins revealed that the assumed tight interaction of either G-domain with Ecdomain 2 that takes place in the GTP conformation always resulted in a decrease of thermostability as compared with the GDP forms. The results provide evidence for the capacity of domain 2 to modulate the state of the G-domain. The mechanism of the modulation was indicated by the CD measurements. The changes in T_ms of the proteins correlated with the changes of their $\theta_{1/2}$ even though the former were absolutely smaller (Table 2). (Values of $\theta_{1/2}$ and T_m cannot be compared directly because they were determined under different experimental conditions, e.g., incubation time; see Materials and Methods.) Our results are consistent with the view that (1) the integrity of the α-helical regions of the G-domain and the function of the protein are closely related; and (2) in the GTP conformation, Ecdomain 2 decreases the thermostability of the G-domain by affecting the stability of its α-helical regions and/or by affecting the stabilizing interactions of the α-helical regions with nonhelical regions of the protein. The substitution of Cys 81 by Gly in EcEF-Tu was shown to abolish this GTP-sensitive phenotype (Anborgh et al. 1992), similarly as in EF-1α, which naturally carries Ala instead of Cys at this position (Nagata et al. 1976). Thus, Cys 81 might be involved in the transmission pathway of a signal from domain 2.

In contrast to EcEF-Tu, the thermostability of BstEF-Tu and CH3, composed of the EcG-domain and Bst domains 2 + 3, was not a function of the guanine nucleotide state, and the stabilizing effect of Bst domains 2 + 3 was not sensitive to the GDP/GTP exchange. All these results indicate that differences exist between Bst and Ec domains 2 in the way they contact and, in turn, modulate (the function of) the G-domain.

The opposing effects of Ec and Bstdomains 2 + 3 on the thermostability in response to GTP also became clearly apparent in experiments aimed at the determination of the GTPase temperature optimum of each protein. The optimum of CH3 was ~10°C higher than that of EcEF-Tu, and vice versa, the GTPase of CH6 had an ~9°C lower temperature optimum than BstEF-Tu (data not shown). Such intermediate thermostability was already reported for a construct composed of the G-domain of archeal *S. solfataricus* and domains 2 + 3 of *E. coli* (Arcari et al. 1999).

The crystal structures of BstEF-Tu · GTP and EcEF-Tu · GTP could help in the identification of interactions involved in the modulation of stability of the G-domain by domain 2. However, in the absence of these crystal structures, a mere correlation between amino acid residues of the G-domain–domain-2 interface, distant up to 4.0 Å and thus likely to be involved in domain interaction in *T. thermophilus* (Tt) EF-Tu · GPPNP (Berchtold et al. 1993), and amino acid residues occurring at homologous positions in the G-domain and domain 2 of Ec and Bst EF-Tus was estab-

lished. The correlation indicates that 21 amino acid residues of the Bst- or EcG-domains might contact, usually more than once, 16 residues in the respective domain 2, and they are, in most cases, identical with amino acid residues of TtEF-Tu · GPPNP. Only in five cases (Fig. 5) did the amino acid residues of these pairs differ between Ec and Bst. These pairs were in Ec/Bst: (1) Ser1/Ala1–Leu264/Leu265; (2) Glu3/Ala3–Leu264/Leu265; (3) Arg7/Arg7–Glu272/Asp273; (4) Arg7/Arg7–Glu267/Gln268; and (5) Arg7/Arg7–Arg269/Glu270. Three of the five pairs listed above involve amino acid residues different (underlined) from those detected in homologous positions of TtEF-Tu · GPPNP. Therefore, their pairing in Bst- or EcEF-Tus is not certain. The remaining two pairs, Arg7–Glu267 (Ec) and Arg7–Asp273 (Bst), also exist in the *T. thermophilus* protein. In Bst, Glu 267 is replaced by Gln 268, and in Ec, Asp 273 is replaced by Glu 272. Thus, at present, consideration of only these structural differences between Ec and Bst domain 2 interfaces, as relevant to different contact properties of Ec and Bstdomain 2, might be justified. Altogether, Ecdomain 2 (residues 200–295) and Bstdomain 2 exhibit ~69% identity in the amino acid sequence, which corresponds to 30 different substitutions. Only three of them are situated at the interface with domain 1 (see above).

Basis for the difference in E. coli
and B. stearothermophilus *G-domain stability*

Differences in ΔG_{stab} as small as 3–6.5 kcal/mole were reported to account for thermostability increases between 10°C and 20°C. This indicates that, in principle, only few changes in the noncovalent forces responsible for ΔG_{stab}
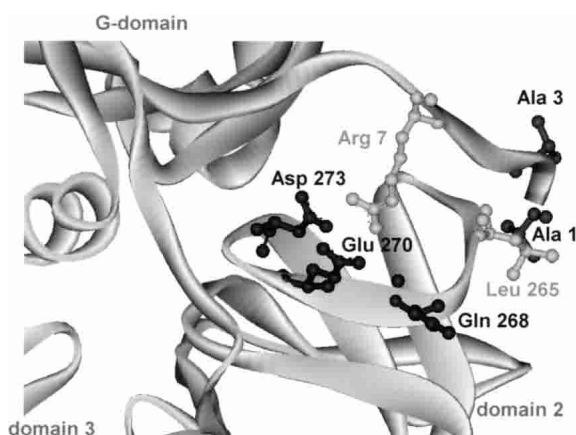

Figure 5. Differences in G-domain–domain 2 interactions between *B. stearothermophilus* and *E. coli* EF-Tus. A homology model of the G-domain–domain 2 interface of *B. stearothermophilus* EF-Tu in GTP conformation. Amino acid residues different from *E. coli* EF-Tu are shown in dark gray; identical residues, in light gray. This figure was made using DS ViewerPro program.

can distinguish mesophilic from thermophilic proteins (for review, see Vieille and Zeikus 2001).

In the absence of the 3D structure of BstEF-Tu, its amino acid sequence was aligned with that of EcEF-Tu to look for the features known to stabilize proteins (Cambillau and Claverie 2000; Vieille and Zeikus 2001).

There are 97 differences in the amino acid structure between both proteins, or the sequence identity is ~75%. As a whole, BstEF-Tu contains three negatively charged residues more and four positively charged residues less than EcEF-Tu. This corresponds with a lower isoelectric point of 4.91 for BstEF-Tu as compared with 5.3 for EcEF-Tu. As shown in our experiments, the G-domains were mainly responsible for the difference in the thermostability between both EF-Tus. However, the G-domains differ from each other at only 38 amino acid positions and are the most similar (81% identity) of all three domains. Nevertheless, the structural features that could lead to different thermal stabilization of the G-domains are prominent. Among 38 amino acid differences, the ratio between charged versus polar, uncharged amino acid residues is 8/11 in the EcG-domain and 14/6 in the BstG-domain. The difference (in percent) between charged and polar uncharged amino acids (*CvP* bias), which has been reported to be the best indicator of the organism's lifestyle (Cambillau and Claverie 2000; Suhre and Claverie 2003), clearly classifies the BstG-domain (*CvP* value = 15.1%) as much more thermostable than the EcG-domain (*CvP* value = 9.5%). According to Cambillau and Claverie plots, the BstG-domain and the EcG-domain "would belong" to the class of hyperthermophiles and moderate thermophiles, respectively. Although this classification provided exaggerated results in absolute terms (see below), the difference in *CvP* values between the G-domains is fully proportional to the difference (~20°C) in thermostability between mesophilic and thermophilic classes of microorganisms. Among amino acid substitutions that occurred between the EcG-domain and the BstG-domain, there were seven replacements by new charged amino acid residues (Gly40Lys, Ala42Glu, Ala57Glu, Pro72Glu, Gln159Glu, Ala186Glu, and Ser197Glu) and seven substitutions of one charged amino acid for another one (Arg44Lys, Lys56Arg, Asp70Glu, Glu155Asp, Asp166Glu, Arg171Lys, and Glu183Lys). The distribution of the charged residues of the BstG-domain different from those present in the EcG-domain is shown on a computer-generated homology model of the BstG-domain (Fig. 6). Most of the replacements by charged residues occurred at the surface of one side of the G-domain, which does not get into contact with domains 2 and 3. The presence of solvent-accessible charged residues at the surface is considered to be a characteristic feature of (hyper)thermophilic proteins because it may allow protein stabilization through ion bonds (Cambillau and Claverie 2000; Suhre and Claverie 2003). The Glu183Lys and Ala186Glu changes are the most likely
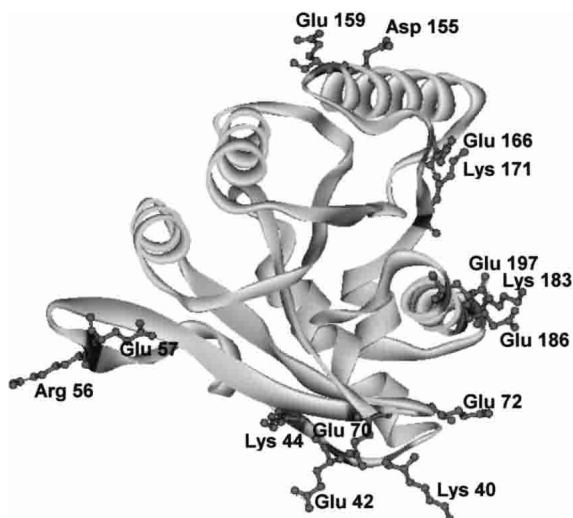
Figure 6. Charged amino acid residues in the G-domain of EF-Tu of *B. stearothermophilus* newly introduced or different from those in the G-domain of EF-Tu of *E. coli*. This figure was made using DS ViewerPro program.

example of an introduction of a new ion pair as *i*, *i* + 3 spacing along an α-helix is consistent with ion pair formation. The same number of charged amino acid residues as in the BstG-domain was found in the G-domain of thermophilic EF-Tu from *T. thermophilus*, and the G-domain of another thermophilic EF-Tu from *Thermus aquaticus* had one additional charged residue.

When the entire molecules of the EF-Tus were subjected to the *CvP*-bias analysis, the values 13.0% for EcEF-Tu and 11.7% for BstEF-Tu were obtained. This was surprising because the reported *CvP*-bias values obtained by the whole-genome analysis of *E. coli* and *B. stearothermophilus* were 2.63% and 9.05%, respectively (Suhre and Claverie 2003), thus fully consistent with the mesophilic character of *E. coli* and the moderately thermophilic character of *B. stearothermophilus*. The discrepancy between the EF-Tu and the whole-genome *CvP* values indicates that both EF-Tu proteins, and EcEF-Tu in particular, significantly differ in the *CvP*-bias features from the majority of cellular proteins and indicates that other strategies, besides the increased ion-pair formation in the G-domain of BstEF-Tu, come into play in the thermostabilization process of the EF-Tu proteins. The *CvP* value of the mesophilic EcEF-Tu being higher than that of the thermophilic BstEF-Tu adds to the recent observation of Suhre and Claviere (2003), demonstrating that the strict correspondence between the highest *CvP* bias and the highest optimal growth temperature breaks down below 80°C. Analysis of *CvP* features of five more members of the EF-Tu family (*B. subtilis*, *T. aquaticus*, *T. thermophilus*, *S. solfataricus*, and *Thermotoga maritima*) provided no simple results either. They will be described elsewhere (H. Šanderová and J. Jonák, in prep.).

B. stearothermophilus is a moderate thermophile, and it has been proposed that in these organisms, the hydrophobic interactions may be greatly involved in the adaptation to high temperatures (Szilagyi and Zavodszky 2000). Indeed, the calculated nonpolar solvent-accessible surface area (Hubbard and Thornton 1993) of the BstG-domain molecule, upon folding, was found to be ~4% smaller than that of EcG-domain · GDP. A similar difference was observed between domains 2 and between domains 3 of both EF-Tus, and the nonpolar solvent-accessible surface area of BstEF-Tu · GDP was calculated to be ~5% smaller than that of EcEF-Tu · GDP. Thus, in contrast to the *CvP* results, a decrease in nonpolar solvent-accessible surface area was detected to occur throughout the entire BstEF-Tu, in all three domains. This implies that an increase in hydrophobic interactions could be an essential reason for the higher thermostability of BstEF-Tu in comparison to EcEF-Tu. Comparative studies between Ec- and BstG-domains revealed that hydrophobic substitutions Val14Ile and Ser65Ala occurred in the core of the BstG-domain. To directly address a possible role of these substitutions in the stabilization of the G-domains, the residues were mutated and the thermostability of the mutated proteins was determined. Four mutants were prepared and examined: BstG-domain Ile14Val, BstG-domain Ala65Ser, EcG-domain Val14Ile, and EcG-domain Ser65Ala. The Ec mutants in the GDP conformation displayed about the same $\theta_{1/2}$, and in the GTP conformation, the Val14Ile mutant had $\theta_{1/2}$ ~2°C higher than the wild-type (wt) EcG-domain. The Bst mutants displayed ~4°–6°C lower $\theta_{1/2}$ in both conformations than the wild-type form of the domain (data not shown). These preliminary experiments at least partially support the involvement of hydrophobic interactions in the stabilization of the G-domains.

The decrease in nonpolar solvent-accessible surface area and a 4% increase in polar solvent-accessible surface area (data not shown) might also be consistent with the observed better solubility of the BstG-domain as compared with the EcG-domain. Whether the change in hydrophobic burial also relates to the slower electrophoretic mobility of BstG-domain/BstEF-Tu in comparison with EcG-domain/EcEF-Tu (Fig. 1) remains to be elucidated.

EF-Tu proteins are unique from the point of view of the division into two moieties, the G-domain and domains 2 + 3, fully unrelated both functionally and structurally. The thermostability of either moiety is considerably lower than the optimum growth temperature of the respective organisms, and the level of thermostability, consistent with it, can only be achieved by the interaction of both moieties in a cooperative way. To elucidate this mechanism, structural–functional studies on more EF-Tus are required to reveal dynamic aspects of the interdomain contacts that have not yet been explained by the static 3D models to date.

Materials and methods

Materials and reagents

The Expand High Fidelity PCR System was purchased from Roche Molecular Biochemicals. The pGEX-5X-3 expression vector, Glutathione Sepharose 4B, reduced glutathione, factor Xa, and [³H]GDP (10 Ci/mmole) were purchased from Amersham Pharmacia Biotech. Phosphoenolpyruvate was obtained from Sigma. 2-Mercaptoethanol, phenylmethyl sulphonylfluoride (PMSF), GDP (Na-salt), and GTP (Na-salt) were from Serva. [γ-³²P]GTP (5000 Ci/mmole) was provided by ICN or Lacomed, and pyruvate kinase was purchased from Calbiochem (Merck). Nitrocellulose membrane filters (HAWP, 0.45 μm; BA85, 0.45 μm) were obtained from Millipore and Schleicher & Schuell Biosciences (Marketing Consulting), respectively. ChromaSpin TE-10 columns were purchased from Clontech (I.T.A.-Intertact).

Bacterial strains and plasmids

Plasmid pEMBL/*tuf*A containing the *E. coli tuf*A gene (Parmeggiani et al. 1987; Jensen et al. 1989) and chromosomal DNA of *B. stearothermophilus* strain CCM 2184 were used as templates for preparation of recombinant and chimeric genes. *E. coli* strain DH5α was used for cloning procedures, and *E. coli* strain BL21 and expression plasmid pGEX-5X-3 were used for overproduction of recombinant proteins.

Construction of recombinant and chimeric genes

The primers 5′-*CGGGATCCCC*TCTAAAGAAAAATTTGAACGTAC-3′ (*Ec*G, forward primer for the *E. coli* G-domain), 5′-*CGAATTC*TTAGCCCAGAACTTTAGCAACA-3′ (*Ec*D3, reverse primer for *E. coli* domain 3), and 5′-*CGGGATCCCC*GCTAAAGCGAAATTTGAGCG-3′ (*Bst*G), and 5′-*CGAATTC*TTACTCGATGATTTCCGATACG-3′ (*Bst*D3) were designed for the amplification of *E. coli* and *B. stearothermophilus tuf* genes for overexpression of recombinant EF-Tu proteins (the flanking regions in italics contain recognition sites of *Bam*HI and *Eco*RI restriction enzymes). The *tuf*A gene of *E. coli* carried by the plasmid pEMBL/*tuf*A (Parmeggiani et al. 1987; Jensen et al. 1989) was used for the preparation, by PCR reaction, of gene fragments coding for the *E. coli* G-domain (EcG-domain, amino acids residues 1–199), Ecdomain 2 (amino acids residues 200–295), Ecdomain 3 (amino acids residues 296–393), as well as the fragments coding for the EcG-domain and Ecdomain 2 (amino acids residues 1–295) and Ecdomains 2 + 3 (amino acids residues 200–393). The chromosomal *tuf* gene of *B. stearothermophilus* was used for the preparation, by PCR reaction, of the gene fragments coding for the *B. stearothermophilus* G-domain (BstG-domain, amino acids residues 1–199), Bstdomain 2 (amino acids residues 200–296), Bstdomain 3 (amino acids residues 297–394), as well as the gene fragments coding for the BstG-domain and Bstdomain 2 (amino acids residues 1–296) and Bstdomains 2 + 3 (amino acids residues 200–394). A further eight synthetic oligonucleotides were designed to synthesize the above fragments of the *tuf*A gene of *E. coli* (Ec) and the *tuf* gene of *B. stearothermophilus* (Bst) for construction of chimeric genes: BstG/EcD2, 5′-*GCGGTTGATGAGTACATC*CCGGAACCAGAGCGTGCGAT-3′ (the flanking region in italics represents the 3′ part of the BstG-domain, and the second part codes for the 5′ part of Ecdomain 2); EcD2/BstG, 5′-*CGCACGCTCTGGTTCCGG*GATGTACTCATCAACCGCGT-3′; BstD2/EcG, 5′-*TTCACGTTGCGGAGTCGG*AATATAA

GAATCCAGGAAGCC-3′; *Ec*G/BstD2, 5′-*TTCCTGGATTCTTATATT*CCGACTCCGCAACGTGAAGT-3′; BstD2/EcD3, 5′-*CAAGTATTGGCAAAACCG*GGCACCATCAAGCCGCACAC-3′; EcD3/BstD2, 5′-*GTGCGGCTTGATGGTGCC*CGGTTTTGCCAATACTTGGC-3′; EcD2/BstD3, 5′-*CAGGTACTGGCTAAGCCG*GGCTCAATCACGCCGCATAC-3′; and BstD3/EcD2, 5-′*ATGCGGCGTGATTGAGCC*CGGCTTAGCCAGTACCTGAC-3′.

The 5′ flanking region (18 nt) of these primers coding for the 3′-end or 5′-beginning of the preceding or following domain, respectively, from the "opposite" gene allowed junction of individual domains to form a template for amplification of chimeric genes. By this procedure, six chimeric forms of EF-Tu, representing all possible combinations of protein domains of EF-Tu from both organisms, were prepared. In addition, free recombinant G-domains of EF-Tu of both organisms were also prepared by overexpression, from the PCR product synthesized using primers 5′-*CGGGATCCCC*TCTAAAGAAAAATTTGAACGTAC-3′ (*Ec*G), 5′-*CGAATTC*TTAAATATAAGAATCCAGGAAGCC-3′ (for EcG-domain), 5′-*CGGGATCCCC*GCTAAAGCGAAATTTGAGCG-3′ (*Bst*G), and 5′-*CGAATTC*TTAGATGTACTCATCAACCGCGT-3′ (for the BstG-domain). Using the *Bam*HI and *Eco*RI restriction sites introduced by primers, the PCR products were cloned in the expression vector pGEX-5X-3 downstream of the recognition site of the serine protease factor Xa. The nucleotide structures of cloned genes were verified by sequencing double-stranded DNA in the ABI PRISM 310 Genetic Analyser. All proteins were produced without N-terminal methionine and with natural stop codons. Because of the cloning in the pGEX vector polylinker and the fusion protein cleavage by factor Xa, the isolated proteins contained three additional N-terminal residues (Gly, Ile, Pro).

Expression and purification of proteins

All recombinant proteins fused with Glutathione S-transferase (GST) were expressed in *E. coli* strain BL21 and separated from the native *E. coli* EF-Tu by affinity chromatography on Glutathione Sepharose 4B using the GST (glutathione-S-transferase) technology. Untagged, GST-free products were obtained after cleavage of the fused proteins with factor Xa, essentially as described in Tomincová et al. (2002).

The GST-G-domain of *E. coli* was isolated by two methods. The soluble fusion protein was purified according to Tomincová et al. (2002) with the following modifications: All buffers contained 10% glycerol; a 100,000*g* supernatant of sonicated cell crude extract was used, and binding of the fusion protein to Glutathione Sepharose 4B was performed on ice for 40 min.

The insoluble fusion protein fraction in inclusion bodies was purified under urea denaturation conditions, essentially as described in Sambrook and Russell (1989).

The concentration of proteins was determined by the procedure of Bradford (1976) using bovine serum albumin as a standard. The concentration of biologically active protein (specific activity) was determined by measuring the maximum binding of [³H]GDP at optimal temperature (Miller and Weissbach 1977). The purity was examined by SDS-PAGE (Laemmli 1970).

Formation of GTP complexes

To obtain proteins in the GTP form, the protein · GDP complex was incubated at 30°C for 20 min (G-domains at 15°C for 8 min) with 4.5 mM phosphoenolpyruvate and 34 mg/L pyruvate kinase. The manufactured GTP and [γ-³²P]GTP were incubated under the

same conditions to eliminate any GDP contamination (Anborgh et al. 1992).

Preparation of nucleotide-free protein

Nucleotide-free proteins were prepared according to Knudsen et al. (1995) using ChromaSpin TE-10 spin columns. Concentration of the active protein in the resulting eluate was determined using the nucleotide binding assay.

Determination of the association and dissociation rate constant and equilibrium dissociation constant of the protein · GDP/GTP complexes

The association rate constant k_{+1} of the [^{3}H]GDP · protein or [^{3}H]GTP · protein complexes was determined by incubating the nucleotide-free protein (5 pmole) and [^{3}H]GDP (5 pmole, specific activity 6000 cpm/pmole) or [^{3}H]GTP (100 pmole, specific activity 8000 cpm/pmole) in 0.5 mL of buffer A (50 mM Tris-Cl at pH 7.6, 10 mM MgCl$_2$, 60 mM NH$_4$Cl, 1 mM DTT) on ice, as described (Fasano et al. 1978; Knudsen et al. 1995). Aliquots (100 μL) were withdrawn at 15, 30, 45, 60, and 75 sec and filtered through a nitrocellulose filter, and the filters were washed with 3 mL of ice-cold buffer A. The results were analyzed according to the second-order rate equation $[1/(b - a)] \cdot \ln[a(b - x)/b(a - x)] = k_{+1}t$, where a is the initial concentration of [^{3}H]GDP/GTP, b the initial concentration of protein, and x the concentration of [^{3}H]GDP · protein or [^{3}H]GTP · protein complexes at time t.

The determination of the dissociation rate constant, k_{-1}, was carried out by incubating 50 pmole of preformed binary complexes of proteins prepared by preincubation with [^{3}H]GDP (500 pmole, specific activity 800 cpm/pmole) or [^{3}H]GTP (5000 pmole, specific activity 4800 cpm/pmole) in 0.5 mL of buffer A on ice for 90 min, essentially as described (Knudsen et al. 1995; Laurberg et al. 1998); the dissociation reaction was started by adding a 1000-fold or 100-fold molar excess of unlabeled GDP or GTP, respectively, in 0.5 mL of buffer A and followed kinetically. Aliquots (100 μL) were withdrawn at regular time intervals and filtered through a nitrocellulose filter; the filters were washed with 3 mL of ice-cold buffer A. The k_{-1} was calculated according to the first-order rate equation $\ln(c_t/c_0) = -k_{-1}t$, where c_0 is the initial concentration of preformed binary complexes and c_t the concentration at different times t.

The apparent equilibrium dissociation constant K_d of the protein · GDP/GTP complexes was determined (Anborgh et al. 1992) by incubating 2 pmole of nucleotide-free protein with 0.5–15 pmole of [^{3}H]GDP (specific activity 2500 cpm/pmole) in 1 mL of buffer A or incubating 50 pmole of nucleotide-free protein with 5–500 pmole of [^{3}H]GTP (specific activity 300–8500 cpm/pmole) in 100 μL of buffer A on ice for 60 min. Then 1 mL or 100 μL from the [^{3}H]GDP or [^{3}H]GTP mixture, respectively, was filtered through a nitrocellulose filter, and the filters were washed as described above. The dissociation constants were calculated using Scatchard plot according to the equation $r/[\text{nucleotide}]_{\text{free}} = 1/K_d(n - r)$, where r is the number of moles of nucleotide bound per mole of protein and n is the number of binding sites (Créchet and Parmeggiani 1986).

Heat stability test

For the heat stability test (Anborgh et al. 1992), protein · GDP or protein · GTP complexes (23 pmole) were incubated in the pres-

ence of 230 pmole of [^{3}H]GDP (specific activity 750 cpm/pmole) or 230 pmole of [γ-^{32}P]GTP (920 pmole in the experiments with EcG-domain; specific activity 2000 cpm/pmole), respectively, in 40 μL of buffer B (50 mM Tris-Cl at pH 7.6; 60 mM NH$_4$Cl, 10 mM MgCl$_2$, 10 mM 2-mercaptoethanol) in the range of 0°–75°C for 8 min, then cooled on ice. Aliquots of 30 μL were spotted on nitrocellulose filters, which were washed three times with 2 mL of cold buffer B. Filters were dried, and the amount of bound nucleotide was measured in a scintillation counter. The temperature-dependent profile of the residual nucleotide binding activity of each protein was determined at least three times.

The circular dichroism (CD) measurement

Measurements were carried out using a JOBIN-YVON CD6 spectrometer in the range of temperatures 10°–75°C. The protein concentration in buffer B was 2.9 μM. Sample cells with 1 mm path-lengths were used. The spectra were accumulated three times at a given temperature. The scanning rate was ~0.3°C/min. The values of molar ellipticity obtained at wavelength 220 nm were plotted against temperature.

Calculations

Solvent-accessible surface areas were calculated using the program NACCESS (Hubbard and Thornton 1993). The default values were used (probe radius, 1.4 Å, z-slices, 0.05 Å, van der Waals radii) in the calculations.

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Paper 3

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Opposite roles of domains 2+3 of *Escherichia coli* EF-Tu and *Bacillus stearothermophilus* EF-Tu in the regulation of EF-Tu GTPase activity

Hana Šanderová, Jiří Jonák*

*Department of Gene Expression, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic,
Flemingovo nám. 2, 166 37 Prague 6, Czech Republic*

Abstract

The effect of noncatalytic domains 2+3 on the intrinsic activity and thermostability of the EF-Tu GTPase center was evaluated in experiments with isolated domains 1 and six chimeric variants of mesophilic *Escherichia coli* (Ec) and thermophilic *Bacillus stearothermophilus* (Bst) EF-Tus. The isolated catalytic domains 1 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 domains 1 differently, depending on the domain origin. Ecdomains 2+3 suppressed the GTPase activity of the Ecdomain 1, whereas those of BstEF-Tu stimulated the Bstdomain 1 GTPase. Domain 1 and domains 2+3 of both EF-Tus positively cooperated to heat-stabilize their GTPase centers to attain optimal activity at a temperature close to the optimal growth temperature of either organism. This can be explained by a stabilization effect of domains 2+3 on α-helical regions of the G-domain as revealed by CD spectroscopy.
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Keywords: Elongation factor Tu; G-domain; GTPase regulation; Thermostability; Chimeric EF-Tu

1. Introduction

Elongation factors Tu (EF-Tu) are three-domain GTPases with an essential function in the elongation phase of mRNA translation. The GTPase center of EF-Tus is in the N-terminal domain (domain 1), called catalytic or G-domain [1]. The G-domain is composed of about 200 amino acid residues, arranged into a predominantly parallel six-stranded β-sheet core surrounded by seven α-helices. Non-catalytic domains 2 and 3 are β-barrels of seven and six, respectively, antiparallel β-strands that share an extended interface. Either non-catalytic domain is composed of about 100 amino acid residues [2,3].

EF-Tu proteins exist in two principal conformations: in a compact one, EF-Tu·GTP, with tight interfaces between all

three domains and a high affinity for aminoacyl-tRNA, and in an open one, EF-Tu·GDP, with essentially no G-domain-domain 2 interactions and a low affinity for aminoacyl-tRNA [4–7]. EF-Tu has approximately a 100-fold higher affinity for GDP than for GTP [8].

The three-domain structure of EF-Tu makes it a model protein for the study of structure-function relationships in multidomain proteins. The binding of GDP or GTP and the GTP hydrolysis are basic activities of all EF-Tus. These activities reside in the G-domain carrying the consensus motif characteristic of all GTP-binding proteins [9]. This was experimentally proved by Parmeggiani et al. [1,10] in experiments with the *Escherichia coli* (Ec) EF-Tu. However, the isolated EcG-domain was found to bind GDP about 1000 times and GTP about 10 times less tightly than the intact three-domain EcEF-Tu. Consequently, the characteristic property of bacterial EF-Tus, the differential affinity for GDP and GTP, was essentially lost.

In our previous experiments we confirmed these observations with the EcG-domain [11,12]. Simultaneously, we

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found out that the G-domain of another EF-Tu, viz. EF-Tu from Gram+ thermophilic *Bacillus stearothermophilus* (Bst), behaved principally differently. Its absolute as well as differential affinities for GDP and GTP were the same as that of the intact BstEF-Tu [11]. As both Ec and Bst full-length EF-Tus bind GDP and GTP with essentially the same (and differential) affinity, our results imply that EcEF-Tu and BstEF-Tu employ a different interdomain strategy to establish the same binding phenotype. While in EcEF-Tu the cooperation involving all three domains of EF-Tu is required, in BstEF-Tu, this binding phenotype is the function of the G-domain itself and it is not distorted by the deletion of domains 2+3.

The molecular mechanism of the other basic function of EF-Tu, the ability to hydrolyze GTP, is still an unresolved issue [13–20] despite the fact that the importance of GTP hydrolysis for the EF-Tu activities was already recognized almost forty years ago [21]. In the present study, we evaluated the role of individual EF-Tu domains in the GTPase reaction. We focused on the intrinsic GTPase to elucidate interdomain relationship in the EF-Tu protein per se. We studied EF-Tu domains from the mesophilic bacterium *E. coli* (EcEF-Tu), and from the thermophile *B. stearothermophilus* (BstEF-Tu). We report here that domains 2+3 of EcEF-Tu and BstEF-Tu have the ability to regulate activity of the GTPase center of their respective G-domains. However, their effects are opposite. Ecdomains 2+3 suppress, whereas Bstdomains 2+3 stimulate the activity of their native GTPase center. On the other hand, domains 2+3 of both EF-Tus increase thermal stability of the GTPase center of the G-domains.

2. Materials and methods

2.1. Materials and reagents

Phosphoenolpyruvate was obtained from Sigma (Prague, Czech Republic). 2-mercaptoethanol, GDP (Na-salt) and GTP (Na-salt) were from Serva (Prague, Czech Republic). [3H]GDP was purchased from Amersham (England). [γ-32P]GTP (5000 Ci/mmol) was provided by ICN (Zlín, Czech Republic) or Lacomed (Řež, Czech Republic). Pyruvate kinase was purchased from Calbiochem (Merck, Říčany, Czech Republic).

2.2. Construction and expression of chimeric and recombinant proteins

Recombinant *E. coli* (Ec) EF-Tu, recombinant *B. stearothermophilus* (Bst) EF-Tu, all chimeric EF-Tu proteins and isolated EcG-domain and BstG-domain were prepared by PCR and GST technologies as described in [11,12]. Composition of chimeric EF-Tus: chimera EBE: Ecdomain 1–Bstdomain 2–Ecdomain 3; chimera EEB: Ecdomains 1–Ecdomains 2–Bstdomain 3; chimera EBB: Ecdomain 1–Bstdomains 2–Bstdomains 3; chimera BEB: Bstdomain 1–Ecdomain 2–Bstdomain 3; chimera BBE: Bstdomains 1–Bstdomains 2–Ecdomain 3; chimera BEE: Bstdomain 1–Ecdomains 2–Ecdomains 3. The biological activity of each protein preparation was determined by measuring its maximum binding of [3H]GDP at optimal temperature [11]. All recombinant proteins, including chimeras, displayed a very similar specific activity in GDP binding in the range of 0.7–1.0 mole of bound GDP/mole of protein, with the exception of the EcG-domain, the activity of which was lower and in the range of 0.07–0.15 mole of bound GDP/mole of G-domain (confer [11]). A low affinity of EcG-domain, prepared by a different method was already described by Parmeggiani et al. [1,10], who prepared the EcG-domain for the first time. The data presented here apply to 100% active proteins. The proteins were stored in 50 mM Tris–HCl, pH 7.6, 100 mM NaCl, 10 mM MgCl$_2$, 1 mM CaCl$_2$, 7 mM 2-ME, 5 μM GDP and 10% glycerol.

2.3. Formation of GTP complexes

The GTP forms of proteins were prepared by incubation of the protein·GDP complex at 30 °C for 20 min (G-domain·GDP at 15 °C for 8 min) with 4.5 mM phosphoenolpyruvate and 34 μg/ml pyruvate kinase. The manufactured GTP and [γ-32P]GTP were preincubated under the same conditions to eliminate any GDP contamination [22].

2.4. GTPase activity

The intrinsic GTPase activity in the presence of 1 M KCl was measured by the liberation of inorganic phosphate from [γ-32P]GTP. Prior to the assay, GDP complexes were converted to GTP complexes as described above. The reaction mixture contained in 65 μl 50 mM imidazolacetate, pH 7.6; 6 mM MgCl$_2$, 74.8 pmol protein·GTP and 1870 pmol [γ-32P]GTP (specific activity 1800 cpm/pmol) or 299 pmol EcG-domain·GTP and 3900 pmol [γ-32P]GTP (specific activity 1800 cpm/pmol), phosphoenolpyruvate and pyruvate kinase taken over with the protein samples and 1 M KCl. The addition of KCl started the reaction, which was followed kinetically at temperatures indicated for 60 min (Fig. 1). Aliquots of 20 μl were withdrawn at appropriate time intervals within the linear course of the reaction and liberated Pi determined by the charcoal method [22]. Blank samples were run simultaneously at every examined temperature to determine background values of GTP hydrolysis and subtracted. The values of hydrolyzed [γ-32P] by EF-Tu proteins were in the range of 17,000–187,500 cpm dependent on increasing temperature after 30 min incubation, while background values without the protein increased from 7000 to 22,000 cpm. The experiments were repeated at least three times in duplicates. The standard deviation was less than 10%. The mean values obtained from the 30 min samples were plotted against
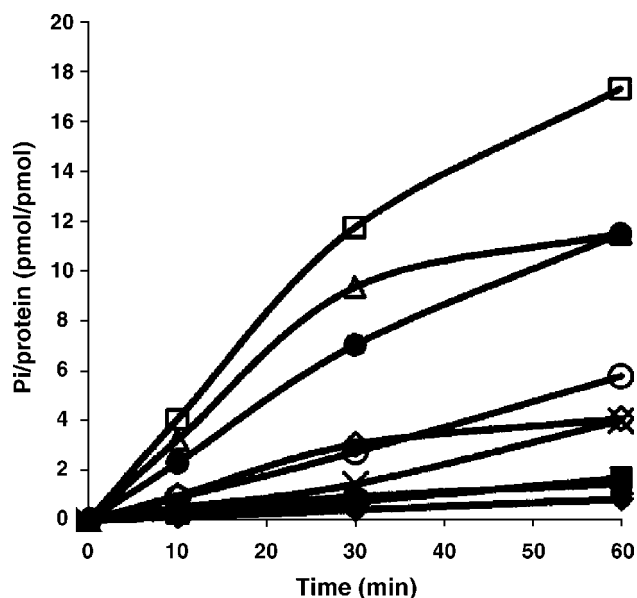
Fig. 1. Time course profiles of the GTPase activity of BstEF-Tu at increasing temperatures. 30 °C (♦), 35 °C (■), 37 °C (▲), 45 °C (x), 50 °C (○), 55 °C (●), 60 °C (□), 65 °C (△), 70 °C (◇).

temperature to obtain GTPase activity profiles of individual proteins. Apparent rate constants were calculated according to [23] (k'_{cat} = mol of [γ-^{32}P]GTP hydrolyzed × mol^{-1} EF-Tu [γ-^{32}P]GTP × s^{-1}).

2.5. The circular dichroism (CD) spectroscopy

Measurements were carried out in the range of temperatures 10–75 °C as described in [11]. The values obtained at 220 nm, indicative of changes in the α-helix content, will be shown.

3. Results and discussion

We applied the domain chimerization approach to elucidate the role of individual EF-Tu domains in the GTPase activity. We used isolated G-domains, full length EF-Tus, and chimeric Ec-Bst and Bst-Ec EF-Tus to assess the contribution of noncatalytic domains 2+3 to the catalytic activity of the G-domain and its thermal stability. This approach is possible because the G-domain and domains 2+3 behave like independent units [1,11,24]. First, we measured GTPase activities of isolated G-domains since they represent the principal functional units of the proteins, and, subsequently, we measured effects of domains 2+3 on the GTPase activity of the G-domain. Table 1 and Fig. 2 summarize the GTPase activities measured starting from protein complexes with [γ-^{32}P]GTP, at high GTP concentrations (29–60 μM), which should ensure near-complete saturation of the proteins so that the measured rates correspond closely to V_{max}. The GTP regeneration system effectively prevented accumulation of EF-Tu·GDP com-

plexes that would inhibit the reaction due to the higher affinity of EF-Tu proteins for GDP than for GTP. The GTP hydrolysis displayed the linear course for at least 30 min at all temperatures that had no denaturing effect on individual proteins (Fig. 1). All the experiments were performed in the presence of 1 M KCl, which enhances the intrinsic GTPase activity [10,23].

In accordance with the high degree of structural identity of the GTPase centers and more than 80% identity in the overall amino acid sequence between EcG-domain and BstG-domain [25], their maximal GTPase activities were, at their optimal temperatures, about the same (Fig. 2, Table 1). However, at 32 °C, the EcG-domain GTPase temperature optimum, the BstG-domain GTPase was about 6-fold less active than the mesophilic one. Conversely, at 50 °C, the BstG-domain GTPase temperature optimum, the activity of the EcG-domain GTPase was about 6-fold less than that of the thermophilic GTPase. Thus, only the temperature ranges in which they are stable and active differentiate the mesophilic from the thermophilic G-domain GTPases. It is very likely that the mechanisms of the increased stabilization of thermophilic GTPase on one side and the "freezing" of its activity at lower temperatures on the other side include an increased number of polar, charged amino acid residues at the expense of polar, uncharged residues and a decrease in the nonpolar solvent accessible surface area in the BstG-domain in comparison to the EcG-domain. We analyzed these features in detail in our previous paper [11].

Next, we investigated the effect of domains 2+3 from either organism on the GTPase activity of their native G-domains. Surprisingly, their effects were opposite; inhibitory by Ecdomains 2+3 and stimulatory by Bstdomains 2+3. The Ecdomains 2+3 although shifting the temperature optimum of the EcG-domain GTPase from 32 °C to 38 °C, depressed its activity in the entire temperature range tested so that it was at most only about 40% as active as in the isolated EcG-domain, in agreement with previous studies conducted at one temperature (confer [1,10,23]). The faster

Table 1
GTPase activity of *E. coli* and *B. stearothermophilus* EF-Tus, G-domains and chimeric EF-Tus

Protein	GTPase temperature optimum (°C)	Specific GTPase activity (mol/mol)	$10^4\ k'_{cat}$ (s^{-1})
Ec G-domain	32	3.5	19.4
Ec EF-Tu	38	1.3	7.2
EBE	44	1.3	7.2
EEB	45	1.5	8.3
EBB	48	1.6	8.8
Bst G-domain	50	4.2	23.3
Bst EF-Tu	61	11.7	65.0
BEB	58	6.4	35.6
BBE	55	8.1	45.0
BEE	51	3.6	20.0

Reaction conditions are described in Materials and methods. The specific GTPase activity was determined as moles of [γ-^{32}P]GTP hydrolysed per mole of protein after 30 min incubation at its temperature optimum.
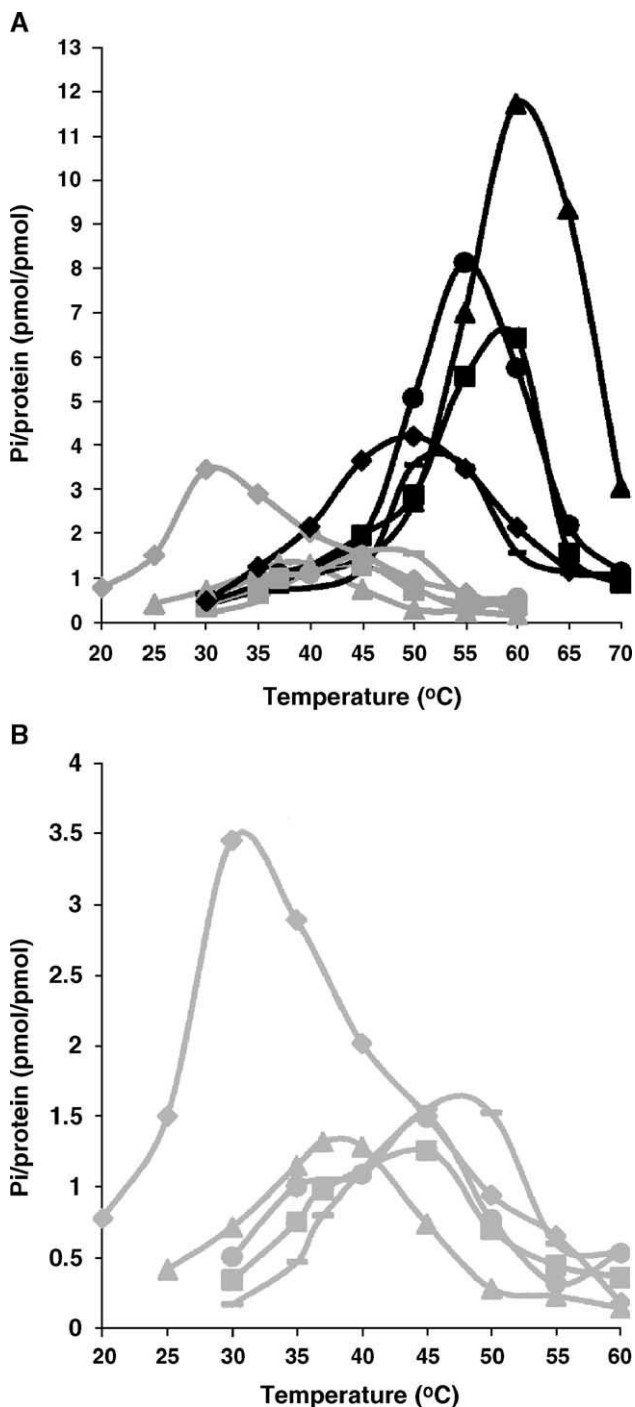
Fig. 2. Effect of temperature on GTPase activity of *E. coli* and *B. stearothermophilus* EF-Tus, G-domains and chimeras. (A) *Ec*EF-Tu (grey ▲), EBE (grey ■), EEB (grey ●), EBB (grey -), *Ec*G-domain (grey ♦), *Bst*EF-Tu (black ▲), BEB (black ■), BBE (black ●), BEE (black -) and *Bst*G-domain (black ♦). (B) Close-up of the thermophilicity of the GTPase of *Ec*EF-Tu, *Ec*G-domain and EBE, EEB and EBB chimeric forms of EF-Tu. The values obtained from 30-min GTPase samples incubated at temperatures indicated are shown.

turnover of the GTPase of the EcG-domain, as compared with EcEF-Tu, was proposed [10] to be a consequence of the isolated EcG-domain's ability to bind GDP and GTP with a similar affinity in contrast to the full-length EcEF-Tu,

which binds GDP with approximately 100-fold higher affinity than GTP.

The effect of the thermophilic Bstdomains 2+3 on the BstG-domain GTPase was also restrictive, but as expected, only at low temperature up to 52 °C, i.e., well below the growth temperature optimum of *B. stearothermophilus*. Above 52 °C, the Bstdomains 2+3 started stimulating the G-domain, similarly as reported for *Sulfolobus solfataricus* EF-1α [26], and at 61 °C, the temperature optimum of BstEF-Tu, the GTPase activity reached about 280% of that of the isolated G-domain. The higher activity of the BstEF-Tu GTPase at its optimal temperature, as compared with the isolated BstG-domain, might be a consequence of the kinetic effect of temperature on the rate of the reaction, as both proteins do not significantly differ in their kinetic parameters of interaction with either GDP or GTP [11]. The switch from the inhibitory to stimulatory effect of Bstdomains 2+3 on the Bst GTPase at ~52 °C correlates with the onset of a change in the CD spectrum at 220 nm, which measures the α-helical content (Fig. 3). This observation suggests that some rearrangement of α-helical regions and/or of α-helical regions stabilizing interactions in the G-domain are required for the stimulatory effect of domains 2+3 to occur.

As expected, the restrictive effect of the thermophilic domains 2+3 on the GTPase activity at low temperatures (up to about 45 °C) was also detected with all chimeric proteins composed of the EcG-domain and one or both Bst noncatalytic domains (Fig. 2B).

The temperature optimum of the EcG-domain GTPase was at 32 °C and that of the BstG-domain GTPase was at 50 °C. The fusion of either G-domain to its non-catalytic domains 2+3 raised the thermal stability and temperature optimum of both EcGTPase and BstGTPase to about 38 °C and 61 °C, respectively, i.e., close to the optimal growth temperature of either organism. The increase in the thermal stability of the EF-Tu GTPases by domains 2+3 could be explained by their stabilization effect on α-helical regions of the G-domains as revealed by CD spectroscopy at 220 nm
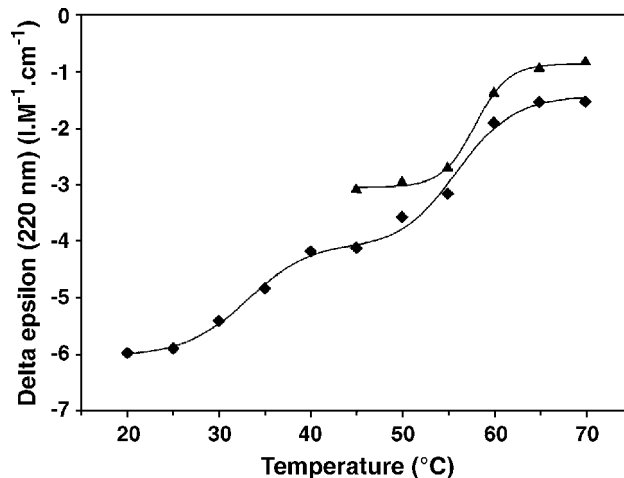

Fig. 3. Thermal unfolding of *Bst*EF-Tu·GTP (▲) and *Bst*G-domain·GTP (♦) monitored by CD spectroscopy.

(Fig. 3). Since α-helices are present only in the G-domain of both EF-Tus, the effect of noncatalytic domains 2+3 on thermal unfolding of the G-domain can be directly examined. It is shown that the temperature-induced changes in α-helical structures of the G-domain within BstEF-Tu·GTP were delayed by about 25 °C as compared to the isolated BstG-domain·GTP itself. Furthermore, the two-step character of the thermal unfolding of the isolated BstG-domain changed into a monophasic one in BstEF-Tu.

Next, to assess whether the inhibitory effect of Ecdomains 2+3 in EcEF-Tu and the stimulatory effect of Bstdomains 2+3 in BstEF-Tu are general properties of these domains, we measured GTPase activities of Ec-Bst and Bst-Ec chimeric proteins (Table 1, Fig. 2). First, we will discuss the effect from the point of view of Ecdomains 2+3. Either of the Ec noncatalytic domains suppressed the EcGTPase activity almost as efficiently as did both Ec noncatalytic domains (compare the activity of EBE or EEB with that of EcEF-Tu). Similarly, either Ec noncatalytic domain was able to decrease the BstGTPase activity (see chimeras BEB, BBE), and their joint effect, as in chimera BEE, decreased its activity even further, below the activity of the isolated BstG-domain. So, regardless of the G-domain's origin, the presence of either or both domains 2+3 of the *E. coli* origin tended to inhibit the GTPase activity of the fused G-domain. Second, we will discuss the effect from the point of view of Bstdomains 2+3. Chimeras composed of BstG-domain, one Bst noncatalytic domain and the other domain of mesophilic origin (BEB and BBE) displayed intermediate activity and stability phenotypes between those of BstG-domain and BstEF-Tu. Given the overall inhibitory effect of either or both Ecdomains 2+3 on both G-domains, we favor the model in which the decreased activity of the chimeras is apparently a consequence of the presence of a noncatalytic Ecdomain. The combination of Bst domains 2+3 raised the GTPase activity of the EcG-domain only by ~20% above that of EcEF-Tu, in stark contrast with the 280% stimulation observed with the BstG-domain. Thus, the stimulatory effect of Bstdomains 2+3 is not universal, and the origin of the G-domain itself, and the G-domain-domains 2+3 mutual interactions appear to play an important role in modulation of the GTPase activity.

Concerning the specificity of the G-domains, we have found out that the same twenty amino acid residues reported in the literature to participate in GDP, GTP and Mg^{2+} ion binding [2–4] and in the GTPase activity [13,15,18,20,27] are all conserved in the G-domain of EcEF-Tu and in BstEF-Tu. Similarly conserved in both EF-Tus are all amino acid residues forming a segment of the polypeptide chain called switch II region (residues 83–89). This segment together with another segment called switch I (residues 40–64) act as sensors for the presence or absence of GTP and transmit this signal from domain 1 to the other two domains [4,28,29]. In the GTP form, the residues at the C-terminal side of the switch I region flank the short α helix α1″, which in the GDP form unwinds to a β-hairpin bridging helix α2 and

domain 3 [28]. By this mechanism, the type of guanine nucleotide directs the conformation and activity of the EF-Tu molecule. In contrast to switch II region, the primary structure of the switch I region differs in 9 positions between EcEF-Tu and BstEF-Tu. We suggest that this difference in the structure of the EF-Tu conformation signaling tools may also be, at least partly, responsible for different signaling effects of domains 2+3 on the G-domain between the mesophilic and thermophilic EF-Tu.

As to the G-domain-domains 2+3 mutual interactions: Since the 3-D structures have not been determined for either *E. coli* or *B. stearothermophilus* EF-Tu in the GTP conformation, a computer-generated homology model of BstEF-Tu·GPPNP was used to assess interdomain contacts [25]. The crystal structure of *Thermus thermophilus* EF-Tu·GPPNP [4] served as a template for constructing the Bst model. The sequence identity between *T. thermophilus* EF-Tu and BstEF-Tu is 78% and between EcEF-Tu and BstEF-Tu is 75%. Therefore, the homology model built can be assumed to be reliable. Several dozens of amino acid pairs identified as forming contacts between Bstdomain 1 and Bstdomain 2 in the BstEF-Tu·GPPNP model were compared with amino acid residues at corresponding positions in the primary structure of EcEF-Tu. Five Ec/Bst differences were detected: S1/A1-L264/L265; E3/A3-L264/L265; R7/R7-E272/D273; R7/R7-E267/Q268; R7/R7-R269/E270. The same approach revealed only one difference in interdomain contacts between Bst and Ecdomains 1 and 3. It is: Ec/Bst-Q124/Q124-R373/K374. Site-directed mutagenesis experiments will be carried out to determine involvement of these amino acid residues in the GTPase regulatory pathways.

Although domains 2+3 are considered to behave as a unit, the chimerization experiments also helped identify individual functions of each of the domain of the complex (Table 1, Fig. 2). It appears that domains 3 of both EF-Tus mainly regulate the thermostability of the proteins: chimeras with the thermophilic domain 3 such as EEB and EBB displayed a higher GTPase temperature optimum than the chimera EBE and similarly, proteins BstEF-Tu and BEB displayed a higher GTPase optimum than chimeras BBE and BEE. On the other hand, domains 2 appear to mainly regulate the GTPase activity: it is higher with the thermophilic domain 2 as it is in BstEF-Tu and BBE compared with BEB or BEE. In agreement with this, mutations in Ecdomain 2 were shown to relieve its negative control behavior resulting in increase in both the intrinsic and the ribosome-dependent GTPase activities of EcEF-Tu [30]. The results with chimeras BBE and BEB also show that either of the mesophilic regulatory domains has the capacity to efficiently support the GTPase at temperatures 55 °C and 58 °C, respectively, which are significantly higher than is the EcGTPase temperature optimum in EcEF-Tu (38 °C).

As shown in Table 1, the optimal GTPase activity of EF-Tu proteins with BstG-domain is decreasing in the order:

BstEF-Tu, BBE, BEB, BEE from 11.7 to 3.6 mol/mol or in their $10^4 \, k_{cat}$ from 65 to $20 \times s^{-1}$. This fully correlates with the GDP-binding parameters of the proteins determined earlier [11]. The correlation indicates that the higher is the GTPase activity of the protein, the higher is the rate constant of GDP dissociation from the protein·GDP complex and the lower is the affinity of the protein for GDP. Thus, the higher GTPase activity might be a consequence of the higher rate of GDP dissociation from the protein GTPase center and vice versa. The GTPase activity of all EF-Tu proteins with EcG-domain is much lower ($1.3–1.6$ mol/mol; $10^4 \, k_{cat}$ $\sim 7.2–8.9 \times s^{-1}$) than that of EF-Tu proteins with BstG-domain and so are differences in the activity of the individual proteins.

In conclusion, the present results demonstrate how the intrinsic GTPase activity and its thermal stability of the two EF-Tu proteins are regulated by their protein domains. These enzymatic experiments, complementing the binding experiments described previously [11], provide evidence for similar and different roles of the non-catalytic domains in the regulation of functions of the two EF-Tu proteins. The Ec and Bst domains $2+3$ appear to be similar in their ability to stabilize both the structure and the functions of their native G-domains against increasing temperature. Moreover, in EcEF-Tu, the domains $2+3$ efficiently neutralize the extreme lability of the isolated EcG-domain [1,11]. Similarly, also in *T. thermophilus* EF-Tu, domains $2+3$ have been reported to play the main stabilizing role for the whole protein and restore capacity to bind nucleotides [31]. On the other hand, Ec and Bst domains $2+3$ differ in their effect on GDP/GTP binding and GTPase activity of the EF-Tus. Ecdomains $2+3$ are absolutely required for establishment of the physiological affinity of EcEF-Tu for GDP and GTP, but they inhibit, put some constraints on, its GTPase activity. In contrast, the physiological affinity of BstEF-Tu for GDP/ GTP appears to be the function of the G-domain itself, domains $2+3$ do not change the nucleotide binding parameters, and they stimulate its GTPase activity. The results imply that EcEF-Tu and BstEF-Tu proteins employ a different G-domain structural strategy and G-domain-domains $2+3$ interaction strategy to establish the same phenotype. In agreement with this, we detected some structural and interaction differences between both proteins. The opposite behavior of domains $2+3$ was not considered before. Even though the effect of domains $2+3$ on the EF-Tu GTPase appears to be small in comparison to the several orders of magnitude larger effect of ribosomes during the physiological mRNA decoding process, we believe that it should be taken into account as an important background whenever the molecular mechanisms of GTPase activation and GTP hydrolysis are being addressed. How the inter-domain effects in EF-Tu are generated, which amino acid residues of the domains are involved, and how these effects of the long range are transmitted between the domains and within domain 1 remain an open question. Their elucidation appears to be a prerequisite for the further understanding of the three-domain character of EF-Tus, alterations in the relative orientation of the domains during translation, as well as the mode of action of potent physiological activators of intrinsic EF-Tu GTPase, such as ribosomes [17]. The findings presented here also extend the list of differences in protein synthesis regulatory mechanisms between Gram-negative and Gram-positive bacteria [11,25,32].

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Paper 4

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The N-terminal region is crucial for the thermostability of the G-domain *of Bacillus stearothermophilus* EF-Tu

Hana Šanderová [a,b], Hana Tišerová [a,b], Ivan Barvík [c], Luděk Sojka [a,b], Jiří Jonák [a,b,d], Libor Krásný [a,b,*]

[a] *Laboratory of Molecular Genetics of Bacteria, Institute of Microbiology Academy of Sciences of the Czech Republic, Vídeňská 1083, Prague 142 20, Czech Republic*
[b] *Department of Bacteriology, Institute of Molecular Genetics Academy of Sciences of the Czech Republic, Vídeňská 1083, Prague 142 20, Czech Republic*
[c] *Institute of Physics, Faculty of Mathematics and Physics, Charles University, Ke Karlovu 5, 121 16 Prague 2, Czech Republic*
[d] *Institute of Medical Biochemistry, First Medical Faculty, Charles University, Kateřinská 32, 121 08 Prague 2, Czech Republic*

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ABSTRACT

Bacterial elongation factor Tu (EF-Tu) is a model monomeric G protein composed of three covalently linked domains. Previously, we evaluated the contributions of individual domains to the thermostability of EF-Tu from the thermophilic bacterium *Bacillus stearothermophilus*. We showed that domain 1 (G-domain) sets up the basal level of thermostability for the whole protein. Here we chose to locate the thermostability determinants distinguishing the thermophilic domain 1 from a mesophilic domain 1. By an approach of systematically swapping protein regions differing between G-domains from mesophilic *Bacillus subtilis* and thermophilic *B. stearothermophilus*, we demonstrate that a small portion of the protein, the N-terminal 12 amino acid residues, plays a key role in the thermostability of this domain. We suggest that the thermostabilizing effect of the N-terminal region could be mediated by stabilizing the functionally important effector region. Finally, we demonstrate that the effect of the N-terminal region is significant also for the thermostability of the full-length EF-Tu.

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1. Introduction

Elongation factor Tu (EF-Tu) is an essential component of the protein translation machinery, and it is the most abundant protein in the bacterial cell. Its main cellular role is to bind aminoacyl-tRNA (aa-tRNA) and deliver it from the cytosol to the A-site of the ribosome during the translation elongation cycle [1].

EF-Tu is a monomeric, three-domain GTP-binding protein that belongs to the large family of G proteins [2]. The N-terminal half of the molecule (approximately 200 amino acid residues) represents domain 1, usually called the G-domain, and it is the catalytic domain containing the binding site for guanine nucleotides and the GTPase center [3]. The structure of the G-domain closely resembles Ras-p21, a protein whose cellular function does not require the presence of additional domains [2]. The other two domains of EF-Tu (2 and 3; ~100 amino acid residues each) have no known catalytic activity, together with the G-domain form the binding site for aa-tRNA [4,5], and modulate, to various extents, the stability, binding and catalytic activity of the G-domain [6,7].

EF-Tu functions as a molecular switch [8,9]. The active form, EF-Tu·GTP, has a compact conformation and displays a high affinity for

aa-tRNA [10]. This ternary complex (EF-Tu·GTP·aa-tRNA), then has a high affinity for the ribosome. After codon–anticodon recognition and hydrolysis of the bound GTP to GDP and inorganic phosphate, the conformation of EF-Tu dramatically changes. The inactive form, EF-Tu·GDP, loses the high affinity for aa-tRNA and dissociates from the ribosome [11–13].

Bacterial EF-Tus are proteins highly homologous in structure and function [1]. Hence, they serve as a perfect model to study how the amino acid sequence reflects the demands of the environment, such as elevated temperatures [14,15].

Our previous comparative studies of EF-Tus from thermophilic *Bacillus stearothermophilus* (temperature growth optimum ~58 °C) and mesophilic *Escherichia coli* (temperature growth optimum ~37 °C) showed that the thermostability of either protein results from cooperative interactions between the G-domain and domains 2 + 3. The G-domains of both EF-Tus establish a basal level of thermostability–substantially higher for the thermophilic than for the mesophilic EF-Tu–and the non-catalytic domains 2 + 3 provide additional stabilization of the G-domains to the level of the optimal growth temperature for either organism. For EF-Tus from both organisms, the extent of the thermostabilizing effect provided by domains 2 + 3 is about the same. The difference in thermostability between the two EF-Tus (~20 °C) is therefore encoded within their G-domains and correlates with the difference in the temperature growth optima between these organisms [6,7]. The G-domain is still relatively large, however, constituting 50% of EF-Tu. It would be highly

informative to identify the region(s) in the G-domain that is responsible for this thermostabilizing effect.

Here, we present a systematic study of the G-domain from *B. stearothermophilus*, in both the GTP and GDP forms, to identify elements important for its thermostability. We compared this G-domain with a corresponding domain from the closely related mesophilic bacterial species *Bacillus subtilis* (temperature growth optimum ~37 °C). We demonstrate that the N-terminal 12 amino acid residues appear to be a strong determinant of the thermostability and integrity of the *B. stearothermophilus* G-domain as well as of the full-length three-domain EF-Tu.

2. Materials and methods

2.1. Materials and reagents

Expand High Fidelity PCR System was purchased from Roche Molecular Biochemicals (Prague, Czech Republic). Glutathione Sepharose 4B, reduced glutathione, factor Xa and [³H]GDP (10 Ci/mmol) were purchased from GE Healthcare (Prague, Czech Republic). Phosphoenolpyruvate was obtained from Sigma (Prague, Czech Republic). 2-mercaptoethanol, phenylmethylsulphonyl fluoride (PMSF), GDP (Na-salt) and GTP (Na-salt) were from Serva (Prague, Czech Republic). [γ-³²P]GTP (5000 Ci/mmol) was provided by MGP (Zlín, Czech Republic) or Lacomed (Řež, Czech Republic), and pyruvate kinase was purchased from Calbiochem (Merck, Říčany, Czech Republic). Nitrocellulose membrane filters (HAWP, 0.45 µm) were obtained from Millipore (Prague, Czech Republic).

2.2. Bacterial strains and plasmids

Chromosomal DNA of *B. stearothermophilus* strain CCM 2184 and chromosomal DNA of *B. subtilis* strain 168 were used as templates to prepare recombinant and chimeric genes. *E. coli* strain DH5α was used for cloning procedures, and *E. coli* strain BL21 and expression plasmids pGEX-1 and pFLAG-CTC were used for overproduction of recombinant proteins.

2.3. Construction of recombinant, chimeric and mutated proteins

Recombinant *B. subtilis* EF-Tu and G-domain, recombinant *B. stearothermophilus* G-domain and all chimeric G-domains were prepared by PCR [6,16] using primers listed in Table 1. Site-directed mutagenesis was performed using QuikChange® Site Directed Mutagenesis Kit from Stratagene. Genes coding *B. subtilis* and *B. stearothermophilus* G-domains, as well as chimeric and mutated G-domains were inserted into expression vector pGEX-1. As this vector contained no protease coding sequence, the cleavage site for factor Xa was inserted into the forward primers just preceding the first codon of the protein. As the factor Xa cleaves after its cleavage site, it enabled preparation of proteins with no additional amino acid residue on the N-terminus. Genes encoding *B. subtilis* and *B. stearothermophilus* G-domains were also inserted into expression vector pFLAG-CTC for expression and preparation of these proteins by a column method (without any tag).

2.4. Purification of recombinant, chimeric and mutated proteins

B. subtilis and *B. stearothermophilus* G-domains, 8 chimeric G-domains, a mutated *B. subtilis* G-domain, *B. subtilis* EF-Tu, and chimeric EF-Tu were purified using GST technology. The detailed protocol is described elsewhere [6,16]. Purified proteins were obtained after cleavage of fusion proteins bound to Glutathione Sepharose 4B by factor Xa [16]. Both G-domains were also prepared by a column method as described elsewhere [17]. All proteins were isolated without the N-terminal Met that is also removed *in vivo*, and all G-domains were extended with 3 amino acid residues Pro–Ile–Pro from the G-domain-domain 2 linker to support the stability of the last

Table 1
List of primers used for construction of chimeric genes, genes for G-domains, and *B. subtilis* EF-Tu.

Name	Sequence	Expression vector
1UF	5′ cg**ggatcc**catcgaaggtcgtgctaaagaaaaattcgaccg 3′	pGEX-1
4UR	5′ c**gaattc**cttgattatggagttgggatgtactc 3′	pGEX-1/pFLAG-CTC
UtufR	5′ g**gaattc**tactattactcagtgattgtagaa 3′	pGEX-1
1SF	5′ cg**ggatcc**catcgaaggtcgtgctaaagcgaaatttgagcgc 3′	pGEX-1
4SR	5′ c**gaattc**cttgattacggagtcgggatgtactc 3′	pGEX-1/pFLAG-CTC
1U2SF	5′ gctgctatcacaacagtacttgcgaaacaagggaaa 3′	
2S1UR	5′ tttcccttgtttcgcaagtactgttgtgatagcagc 3′	
2S3UF	5′ cacgtcgagtatgaaacagaaactcgtcactatgca 3′	
3U2SR	5′ tgcatagtgacgagtttctgtttcatactcgacgtg 3′	
2U3SF	5′ cacgttgagtacgaaactgaggctcgtcactacgcg 3′	
3S2UR	5′ cgcgtagtgacgagcctcagtttcgtactcaacgtg 3′	
3S4UF	5′ gaagtgccggttatcaaaggttctgctcttaaagct 3′	
4U3SR	5′ agctttaagagcagaacctttgataaccggcacttc 3′	
3U4SF	5′ gatgtaccagttgttaaaggttcggcattaaaagcg 3′	
4S3UR	5′ cgcttttaatgccgaaccttaacaactggtacatc 3′	
1S2UF	5′ gctgcgatcacgacggttcttcataagaaatctggt 3′	
2U1S1	5′ accagatttcttatgaagaaccgtcgtgatcgcagc 3′	
1UF	5′ gggccgg**catatg**gctaaagaaaaattcg 3′	pFLAG-CTC
1SF	5′ gggccgg**catatg**gctaaagcgaaatttg 3′	pFLAG-CTC

The nomenclature of the primers is based on the identity of the region (1 = N-terminal region; 2 = effector region; 3 = region between regions 2 and 4; 4 = C-terminal region) and on the origin of the region (U = region from *B. subtilis*, S = region from *B. stearothermophilus*). Primers for amplification of chimeric regions contained two parts: the 5′ flanking region (in *italics*) coding for a region from one G-domain and the 3′ region coding for the downstream region from another G-domain. F = forward primer; R = reverse primer. The restriction sites used for insertion of genes into indicated expression vectors are in bold. The fragment encoding the factor Xa proteolytic site is underlined.

α-helix of the G-domain. All proteins were purified to at least 95% homogeneity as determined by SDS-PAGE [18]. The biological activity of each protein preparation was determined by measuring its maximum binding of [³H]GDP at optimal temperature [6]. All recombinant proteins displayed a specific activity in GDP binding in the range of 0.3–0.9 mol of bound GDP/mole of protein. The proteins were stored in 50 mM Tris-Cl, pH 7.6, 100 mM NaCl, 10 mM MgCl₂, 1 mM CaCl₂, 7 mM 2-ME, 5 µM GDP and 10% or 50% glycerol.

2.5. Functional thermostability tests

GDP and GTP binding assays at increasing temperature were performed as described by Sanderova et al [6]. The GTPase activities were tested as described elsewhere [7].

2.6. Differential scanning calorimetry

Measurements were taken using a VP-DSC instrument in the range of temperatures 5 °C–100 °C and with a scanning rate of 1 °C/min. Protein concentration in the buffer (20 mM Tris, pH 7.6, 1 mM MgCl₂; 50 µM GDP) was 45 µM. The exact protein concentration was determined by amino acid analysis. The data were analyzed using software developed by MicroCal (MicroCal, Northampton, MA, USA).

2.7. Calculations

Solvent accessible surface areas were calculated using the program NACCESS [19]. The default values were used (probe radius, 1.4 Å, z-slices, 0.05 Å, van der Waals radii) in the calculations.

3. Results and discussion

3.1. G-domain sets up basal level of thermostability of EF-Tu from B. subtilis

In our previous report, we used EF-Tus from mesophilic *E. coli* and thermophilic *B. stearothermophilus* as model proteins. A comparison
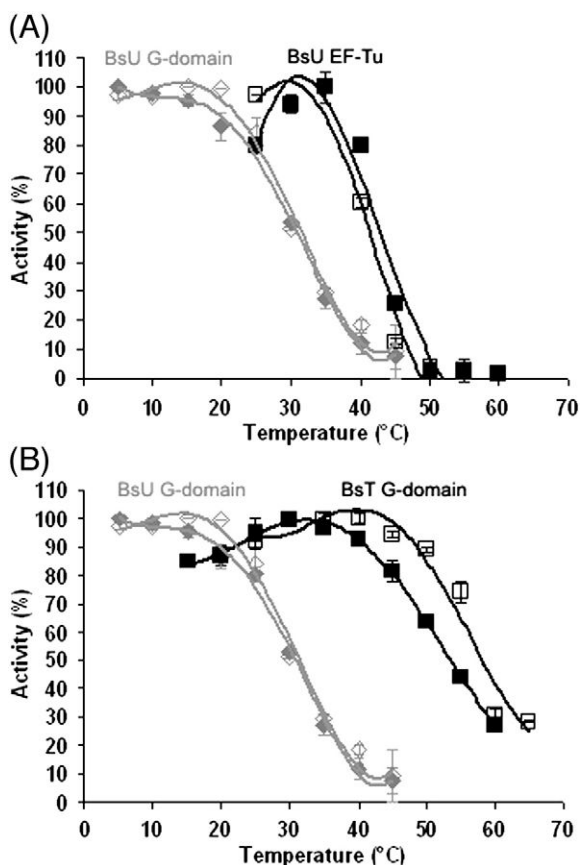
Fig. 1. Heat inactivation profiles of GDP (closed symbols) and GTP (open symbols) forms of (A) *B. subtilis* G-domain (in gray) and *B. subtilis* EF-Tu (in black) and (B) *B. subtilis* (in gray) and *B. stearothermophilus* (in black) G-domains. The error bars in this and all other figures represent ± SD of the mean.

of the EF-Tu G-domains from these organisms revealed 38 differences in amino acid sequence [6,7]. To simplify the study of the thermostability determinants in EF-Tu, we searched for a mesophilic

organism that has fewer amino acid substitutions in the EF-Tu G-domain. We identified *B. subtilis*, a widely used model organism that contains only 22 amino acid substitutions in the G-domain in comparison with the *B. stearothermophilus* G-domain [20,21].

First, to establish whether EF-Tu from *B. subtilis* is a suitable mesophilic model protein, we determined its thermostability in both GDP and GTP forms by measuring their heat inactivation profiles (Fig. 1A). The temperatures at which half of the EF-Tu's protein activity was lost ($\theta_{1/2}$) were 42.9 °C and 41.5 °C for the GDP and GTP forms, respectively. These values closely resembled those determined for EF-Tu from *E. coli* [6].

Second, we prepared a recombinant G-domain of *B. subtilis* EF-Tu, determined its $\theta_{1/2}$ values for GDP and GTP forms, and compared these values with the corresponding values of the full-length protein (Fig. 1A). Similarly to our previous results with G-domains and EF-Tus from *E. coli* and *B. stearothermophilus*, the removal of noncatalytic domains 2 + 3 decreased the thermostability of the G-domain (by ∼10 °C to 31 °C and 31.5 °C for GDP and GTP form, respectively). This suggests that the thermostability of *B. subtilis* EF-Tu is also a result of cooperative interactions between the G-domains and domains 2 + 3.

Our data showed (i) that EF-Tu from *B. subtilis* is a suitable mesophilic model protein for comparative studies of thermostability determinants, and (ii) that its G-domain appears to establish the basal level of thermostability for the full-length EF-Tu, similarly to EF-Tus from *E. coli* and *B. stearothermophilus*.

3.2. G-domains from B. stearothermophilus and B. subtilis differ in thermostability

Next, while focusing on their thermostability, we compared the properties of *B. stearothermophilus* and *B. subtilis* G-domains in several tests.

First, we compared their activities and stabilities in functional tests: (i) GDP binding, (ii) GTP binding, and (iii) GTPase activity. As shown in Fig. 1B and Table 2, the G-domains' $\theta_{1/2}$ differed by 22.8 °C (GDP binding) and 26.6 °C (GTP binding). The difference between their GTPase activity temperature optima was about 20 °C (data not shown).

Second, the structural stability of both G-domains in GDP forms was determined by differential scanning calorimetry (DSC). This

Table 2
Comparison of the thermal stabilities of G-domains and G-domain chimeras.

Protein	$\theta_{1/2}$ (GDP) [°C]	$\Delta \theta_{1/2}$ (GDP) compared to BsU G-domain [°C]	$\theta_{1/2}$ (GTP) [°C]	$\Delta \theta_{1/2}$ (GTP) compared to BsU G-domain [°C]
(A)				
BsU G-domain	31.0		31.5	
1U-2S-3U-4U	26.2	− 4.8	32.5	+ 1.0
1U-2U-3U-4S	32.8	+ 1.8	34.7	+ 3.2
1U-2U-3U-4M	34.0	+ 3.0	33.8	+ 2.3
1S-2U-3U-4U	41.0	+ 10.0	40.2	+ 8.7
1S-2S-3U-4U	43.8	+ 12.8	42.3	+ 10.8

Protein	$\theta_{1/2}$ (GDP) [°C]	$\Delta \theta_{1/2}$ (GDP) compared to BsT G-domain [°C]	$\theta_{1/2}$ (GTP) [°C]	$\Delta \theta_{1/2}$ (GTP) compared to BsT G-domain [°C]
(B)				
BsT G-domain	53.8		58.1	
1S-2U-3S-4S	54.1	+ 0.3	50.8	− 7.3
1S-2S-3S-4U	50.0	− 3.8	47.0	− 11.1
1U-2S-3S-4S	41.3	− 12.5	42.2	− 15.9
1U-2U-3S-4S	48.0	− 5.8	47.1	− 11.0

Protein	$\theta_{1/2}$ (GDP) [°C]	$\theta_{1/2}$ (GTP) [°C]
(C)		
BsU EF-Tu	42.9	41.5
1S-UEF-Tu	48.1	48.8

(A) Comparison of the *B. subtilis* (BsU) G-domain with chimeras of *B. subtilis* G-domain origin containing regions from the *B. stearothermophilus* G-domain, (B) comparison of the *B. stearothermophilus* (BsT) G-domain with chimeras of *B. stearothermophilus* G-domain origin containing regions from the *B. subtilis* G-domain, and (C) parameters characterizing the thermal stability of *B. subtilis* EF-Tu (BsU EF-Tu) and a chimera (1S-UEF-Tu) containing the N-terminal region from *B. stearothermophilus* EF-Tu and the rest of the molecule from *B. subtilis* EF-Tu. The differences in thermostabilities of chimeras and respective G-domains are shown. $\theta_{1/2}$ = temperature at which half of the protein sample is inactivated; $\Delta \theta_{1/2}$ values: $\theta_{1/2}$ of the indicated G-domain subtracted from $\theta_{1/2}$ of given chimera; (+) = increased thermostability, (−) = decreased thermostability. This value reflects the thermo(de-)stabilizing effect of a region from one organism in the context of the rest of the molecule from the other organism. See text for a description of the chimeras.

method monitors the difference in the amount of heat required to increase the temperature of a sample and reference as a function of temperature. The difference in transition midpoints (T_ms), when 50% of the biomolecules are unfolded, was 18.3 °C between the two G-domains (data not shown).

Subsequently, a bioinformatic analysis was performed to identify protein properties that could explain the observed 20 °C difference in thermostability between the two proteins. The ratio between charged versus polar uncharged amino acid residues (CvP bias) [22,23] and the solvent accessible surface area [19] revealed that the *B. stearothermophilus* G-domain displayed a slightly higher CvP value than the *B. subtilis* G-domain (14.3 versus 13.3) and a 2% lower nonpolar solvent accessible surface area in both GDP and GTP conformations. Both these characteristics are consistent with the higher thermostability of the *B. stearothermophilus* G-domain. The differences are very small, however, thus reflecting the close phylogenetic relationship between the two proteins. In fact, these values by themselves would not indicate a difference in thermo-stability of 20 °C.

We concluded that G-domains from *B. stearothermophilus* and *B. subtilis* differed by about 20 °C in their thermostabilities. In all experiments, the *B. stearothermophilus* G-domain was consistently more thermostable than the *B. subtilis* G-domain. The difference in thermostability of the two G-domains correlated with the difference in thermostability of the respective EF-Tus, which correlated with the optimal growth temperatures of the two organisms. Our bioinfor-matic analysis (CvP bias and solvent accessible surface area) did not provide an obvious explanation for the higher thermostability of the *B. stearothermophilus* G-domain and thus underscored the need for a direct experimental approach.

3.3. Comparison of amino acid sequences of B. subtilis and B. stearothermophilus G-domains

In our experimental setup the engineered *B. subtilis* G-domain was composed of 203 amino acid residues and the *B. stearothermo-philus* G-domain of 202 amino acid residues. Polypeptide chains of both G-domains started with the N-terminal Ala and ended with the
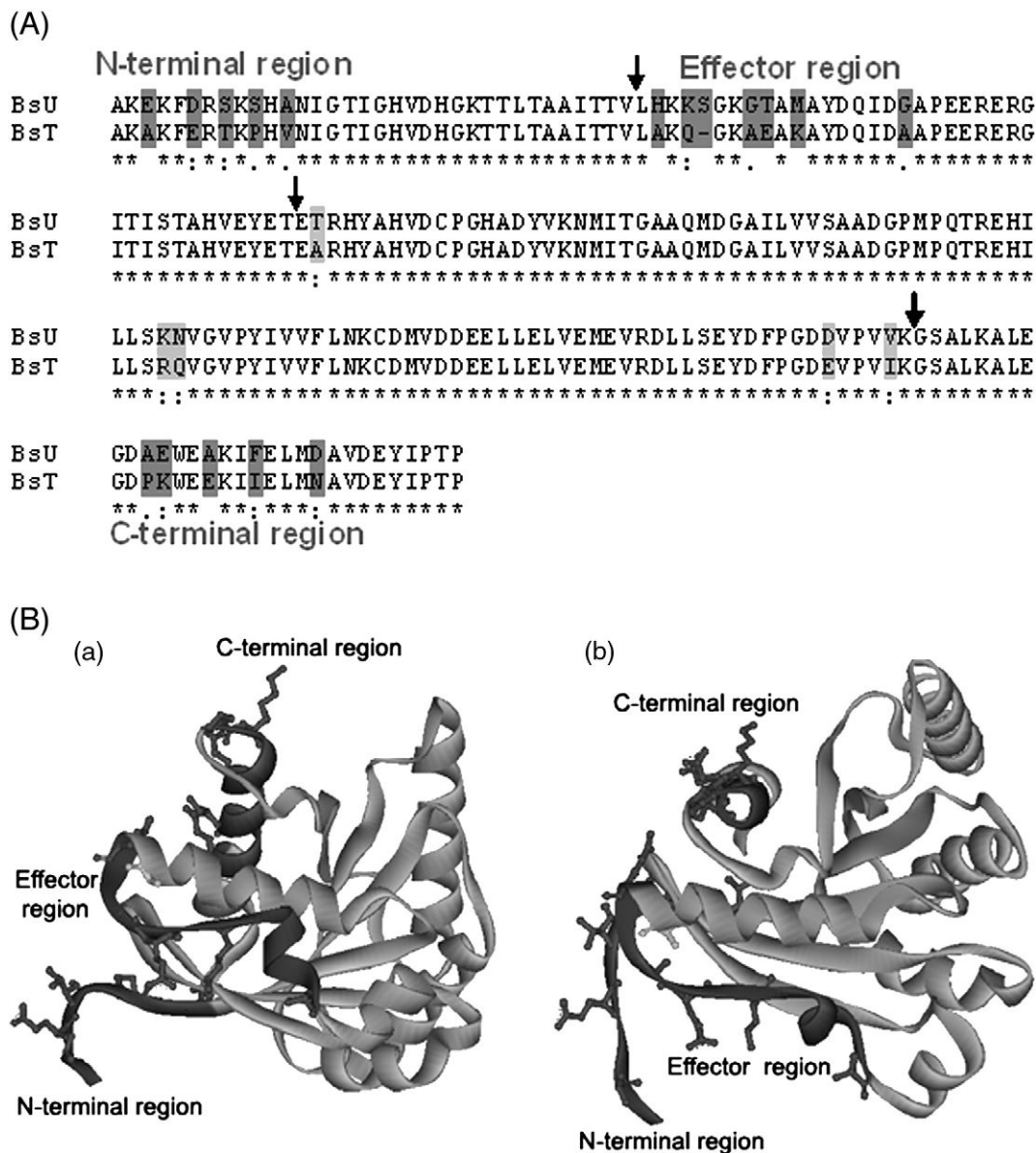

Fig. 2. Comparison of *B. subtilis* and *B. stearothermophilus* G-domains. (A) Alignment of amino acid sequences of *B. subtilis* and *B. stearothermophilus* G-domains. Different amino acid residues are highlighted. The arrows divide the G-domain into four regions that were combined to create the chimeras. (B) Homology models of 3D structure of *B. stearothermophilus* G-domain with marked regions containing different amino acid residues in comparison with *B. subtilis* G-domain. (a) a front view, (b) a top view.

C-terminal Pro. The *B. subtilis* G-domain carried an additional amino acid, Ser, at position 39 (Fig. 2A).

Alignment of amino acid sequences of G-domains from *B. stearothermophilus* and *B. subtilis* revealed a high, 89% identity in their primary structures. The two G-domains differ in only 22 out of 203 amino acid residues. As these two G-domains differed by about 20 °C in thermostability, these 22 amino acid residues should encode this difference.

The alignment showed that most of the 22 different amino acid residues are located in three distinct regions. We designated these regions according to their localization as (i) the N-terminal, (ii) effector, and (iii) C-terminal region (Fig. 2A). A homology 3D model of the *B. stearothermophilus* G-domain revealed that most of these amino acid residues are positioned on the surface of the molecule, facing the solvent (Fig. 2B).

Based on these observations, we decided to construct chimeric G-domains containing various combinations of these regions from *B. subtilis* and *B. stearothermophilus* G-domains and to analyze their thermostabilities. The strategy of chimera design was based on reciprocal swapping of the selected regions. The nomenclature of the chimeric G-domains was based (i) on the position of the region within the G-domain (1 = N-terminal region; 2 = effector region; 3 = region between regions 2 and 4; 4 = C-terminal region), and (ii) on the origin of the region (U = region from *B. subtilis*, S = region from *B. stearothermophilus*). For example, the chimera 1U-2S-3U-4U was composed of regions 1, 3 and 4 from *B. subtilis*, and region 2 was from *B. stearothermophilus*.

As the main experimental technique to evaluate the thermostabilities of all created chimeric proteins we selected functional tests by GDP and GTP binding assays (comparing $\theta_{1/2}$ values). As it is easier to change a protein to decrease its stability, we were primarily interested in changes in the *B. subtilis* G-domain that increased its stability.

3.4. Effector region

The effector region was named after its function in Ras-p21 where it interacts with effector molecules [2]. The effector region is also called the switch I region in EF-Tu proteins and it can be divided into two parts. The N-terminal part of the effector region of EF-Tu (amino acid residues 40–48; *B. stearothermophilus* numbering) is the most variable part of bacterial EF-Tus and, conversely, the C-terminal part (amino acid residues 53–59) is well conserved among prokaryotic EF-Tus [4,8]. The effector region of EF-Tu undergoes a profound conformational change after hydrolysis of GTP when the small α-helix is unwound to β-sheet [4,8,24]. We were interested in the variable N-terminal part of the effector region plus several preceding amino acid residues (residues 36–51 in *B. stearothermophilus* EF-Tu) that are positioned on the surface of the molecule and where the two studied G-domains differ in 7 amino acid residues (Fig. 2).

In the GDP conformation, the effector region from *B. stearothermophilus* had no positive effect on the thermostability of the *B. subtilis* G-domain as tested with the 1U-2S-3U-4U chimera (Table 2A, Fig. 3A). In fact, it even destabilized, by 4.8 °C, this chimera in comparison with the *B. subtilis* G-domain. Similarly, the *B. subtilis* effector region (1S-2U-3S-4S) did not have a significant effect on the G-domain that was otherwise from *B. stearothermophilus*: This change very slightly (but reproducibly) thermostabilized the 1S-2U-3S-4S chimera in comparison with the *B. stearothermophilus* G-domain (Table 2B, Fig. 3A).

In the GTP conformation, the effector region from *B. stearothermophilus* within the 1U-2S-3U-4U chimera stabilized the *B. subtilis* G-domain only slightly, by 1.0 °C (Table 2A, Fig. 3B). On the other hand, the *B. subtilis* effector region destabilized the 1S-2U-3S-4S chimera by 7.3 °C (Table 2B, Fig. 3B).

We concluded that the *B. stearothermophilus* effector region did not significantly stabilize the *B. subtilis* G-domain within the 1U-2S-3U-4U chimera in either conformation. The effect of the *B. subtilis* effector
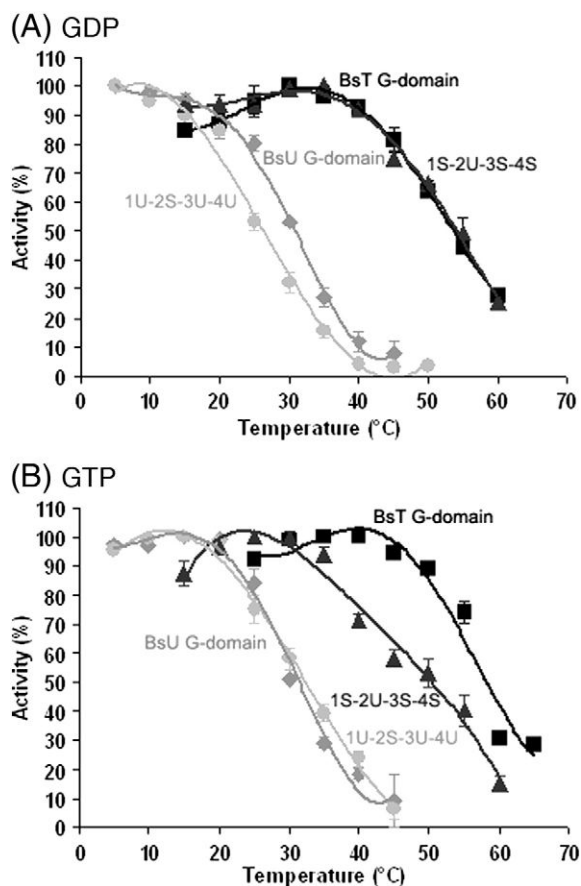

Fig. 3. Effect of the effector region. Heat inactivation profiles of (A) GDP and (B) GTP forms of *B. subtilis* G-domain (gray diamonds), chimera 1U-2S-3U-4U (light gray circles), *B. stearothermophilus* G-domain (black squares) and chimera 1S-2U-3S-4S (dark gray triangles).

region within the *B. stearothermophilus* G-domain in chimera 1S-2U-3S-4S was different: in GDP conformation it (surprisingly) did not destabilize the chimera, whereas in GTP conformation it did. We can speculate that this difference may have been caused by different interactions of the *B. subtilis* effector region with the rest of the molecule in either conformation. In summary, no clear trend was observed in these experiments, and the effector region appeared to have no significant thermostabilizing effect by itself.

3.5. C-terminal region

The C-terminal region contains 5 amino acid differences within the last F-helix of the G-domain (Fig. 2). Again, these amino acid residues are positioned on the surface of the molecule and have no contacts with other domains of EF-Tu in either conformation.

G-domains with the swapped C-terminal regions revealed that the C-terminal region from *B. stearothermophilus* increased thermostability of the *B. subtilis* G-domain by 1.8 °C and 3.2 °C in the GDP and GTP forms, respectively, as tested using the 1U-2U-3U-4S chimera (Table 2A, Fig. 4). The C-terminal region from *B. subtilis* within the reciprocal 1S-2S-3S-4U chimera destabilized the *B. stearothermophilus* G-domain by 3.8 °C and 11.1 °C in the GDP and GTP forms, respectively (Table 2B, Fig. 4).

To evaluate the effect of this region on thermostability in more detail, we prepared a *B. subtilis* G-domain mutant (1U-2U-3U-4M) containing just 3 amino acid residues from the *B. stearothermophilus* G-domain (Ala184Pro, Glu185Lys, Ala188Glu). These *B. stearothermophilus* amino acid residues were selected since they differ from homologous amino acid residues that are the same in mesophilic *E.*
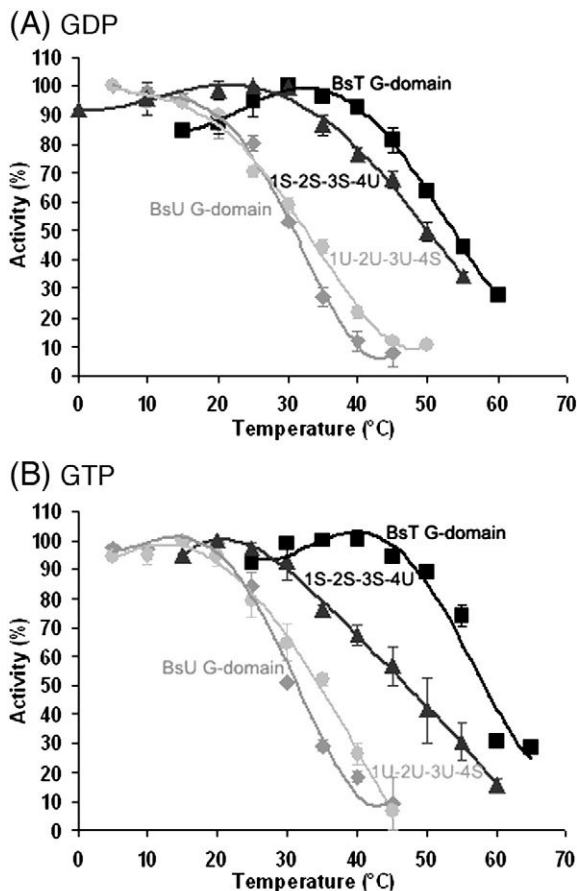
Fig. 4. Effect of the C-terminal region. Heat inactivation profiles of (A) GDP and (B) GTP forms of *B. subtilis* G-domain (gray diamonds), chimera 1U-2U-3U-4S (light gray circles), *B. stearothermophilus* G-domain (black squares) and chimera 1S-2S-3S-4U (dark gray triangles).

coli and *B. subtilis* G-domains. The thermostabilities of this mutant in GDP and GTP conformations were similar to those of the 1U-2U-3U-4S chimera (Table 2A).

We concluded that the *B. stearothermophilus* C-terminal region had a moderate, positive effect on the thermostability of the *B. subtilis* G-domain and that this effect was caused by just three amino acid residues in this region.

We furthermore applied molecular dynamic (MD) simulations to both G-domains (not shown). In the absence of 3D structures for EF-Tus and G-domains from *B. subtilis* and *B. stearothermophilus*, homology models of both G-domains were prepared based on experimentally determined 3D structures of EF-Tus from *E. coli* and *Thermus thermophilus*. The MD simulations showed that the *B. stearothermophilus* C-terminal region may be more rigid and, therefore, more resistant to denaturation by heat than the corresponding region from *B. subtilis*. This is due to the presence of a proline residue localized in this region (Pro182 in *B. stearothermophilus*–Ala183 in *B. subtilis*). It had been described previously that thermophilic proteins have higher frequency of proline residues in their structures. Proline residues have the lowest conformational entropy and, therefore, increase the rigidity of protein conformations [15,25].

3.6. N-terminal region

The conserved N-terminal region amino acid residues Lys 4 and Lys9 of *E. coli* EF-Tu have been shown to be important for aa-tRNA binding but not to have a large effect on thermostability [26–28].

In our study, the N-terminal regions are represented by the first 12 amino acid residues of the G-domains. They differ in 5 amino acid

residues between *B. subtilis* and *B. stearothermophilus* G-domains (Fig. 2). The position of the N-terminal region is not clearly defined in the known 3D structures of EF-Tu proteins. In the GDP conformation of EF-Tus [5,8,24], this region is usually missing since it is flexible and not captured by the electron density maps; in the 3D structures of EF-Tu in GTP conformation [4,29] it seems that it binds to domain 2. In 3D structures of the EF-Tu·GDPNP·aa-tRNA ternary complex [30,31], meanwhile, this region is positioned in the cleft between the G-domain and domain 2. These findings suggest that in each of these forms, the N-terminal region may bind in a different position. As a consequence, we expected no profound effect on the thermostability of the G-domain from the swapping of this part of the molecule between the two G-domains.

Surprisingly, the N-terminal region from *B. stearothermophilus* stabilized the *B. subtilis* G-domain by 10.0 °C and 8.7 °C in the GDP and GTP conformations, respectively, as tested using the 1S-2U-3U-4U chimera (Table 2A, Fig. 5). Accordingly, the reciprocal chimera (1U-2S-3S-4S) was by 12.5 °C and 15.9 °C (GDP and GTP forms) less thermostable than the *B. stearothermophilus* G-domain (Table 2B, Fig. 5).

To further evaluate the effect of the N-terminal region on thermostability, we intended to prepare truncated forms of both G-domains that would lack various portions of the N-terminal region. First, we prepared DNA constructs for expression of both G-domains without 9 N-terminal residues that seemed to be located outside of the compact 3D structure of the G-domains. Second, we prepared DNA constructs for expression of both G-domains without 12 N-terminal amino acid residues that included all 5 amino acid differences between both G-domains. Unfortunately, already at the level of
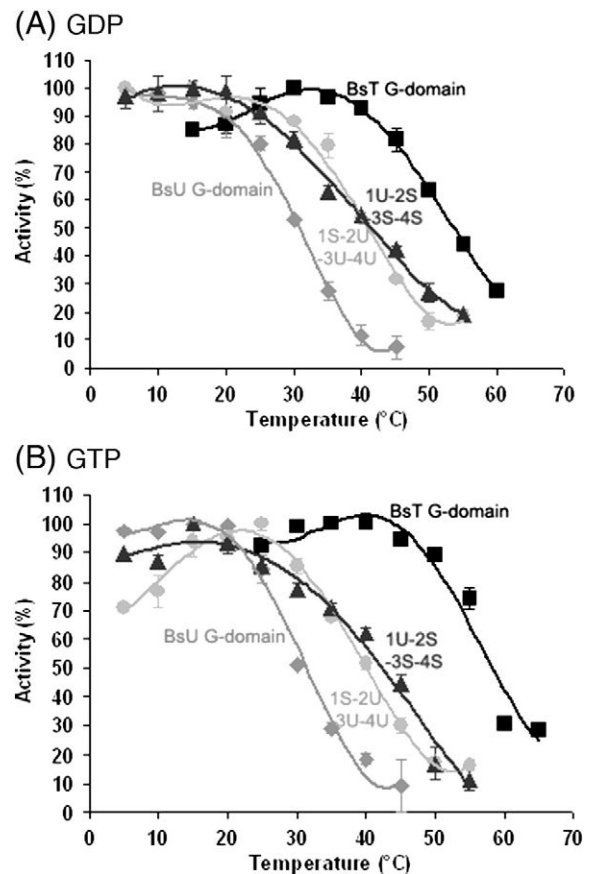

Fig. 5. Effect of the N-terminal region. Heat inactivation profiles of (A) GDP and (B) GTP forms of *B. subtilis* G-domain (gray diamonds), chimera 1S-2U-3U-4U (light gray circles), *B. stearothermophilus* G-domain (black squares) and chimera 1U-2S-3S-4S (dark gray triangles).
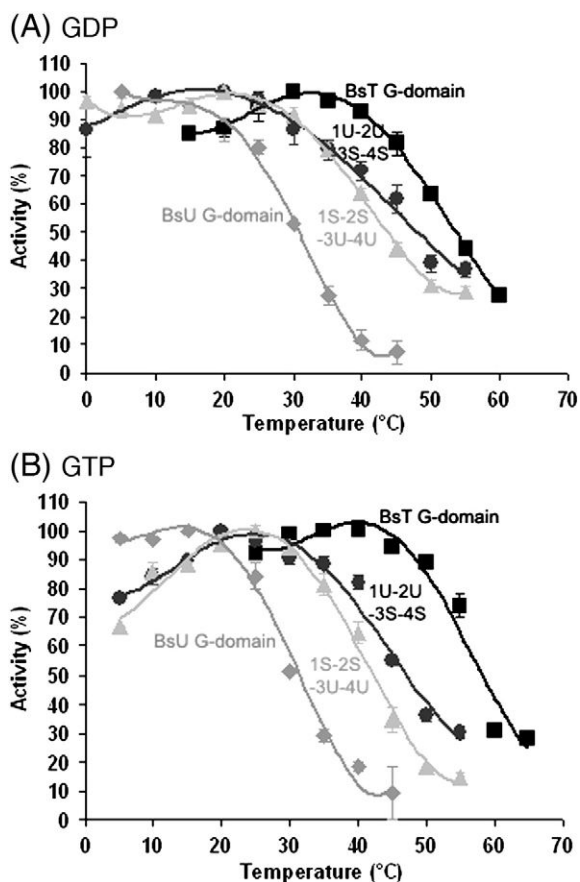
Fig. 6. Effect of the N-terminal and effector regions. Heat inactivation profiles of (A) GDP and (B) GTP forms of *B. subtilis* G-domain (gray diamonds), chimera 1S-2S-3U-4U (light gray circles), *B. stearothermophilus* G-domain (black squares) and chimera 1U-2U-3S-4S (dark gray triangles).

expression of these genes, we were able to detect only negligible amounts of these proteins in the cell, suggesting that their biological stabilities were seriously affected. Understandably, several attempts to purify these proteins failed due to the minimal amount of the starting material. This negative result further underscored the importance of the N-terminal region of the G-domain.

We concluded that the N-terminal 12 amino acid residues appeared to be critical determinants of the G-domain thermostability for *B. stearothermophilus* EF-Tu. Moreover, the unsuccessful attempts to purify the *B. subtilis* and *B. stearothermophilus* G-domains lacking this region indicated that these 12 amino acid residues may be critical for the biological stability of the G-domains *in vivo*.

3.7. The N-terminal and effector region may cooperate to stabilize G-domain

The next question we wished to answer was how the N-terminal region stabilizes the G-domain. Previously, it had been suggested that the N-terminal region may interact with the effector region [26], and we hypothesized that this interaction may be important for the thermostability of the G-domain. We decided to test this hypothesis by using reciprocal chimeras that contained the N-terminal and effector regions from one organism in the context of the rest of the G-domain from the other organism.

As shown in Fig. 6 and Table 2A, this double substitution by the homologous regions from *B. stearothermophilus* (chimera 1S-2S-3U-4U) increased the thermostability of the *B. subtilis* G-domain by 12.8 °C and 10.8 °C in GDP and GTP conformations, respectively. This stabilization was ~25% more pronounced than the stabilization

provided by the N-terminal region alone. As the *B. stearothermophilus* effector region alone did not significantly stabilize the *B. subtilis* G-domain, the detected 25% increase in thermostability suggested some cooperation between the two *B. stearothermophilus* regions. The

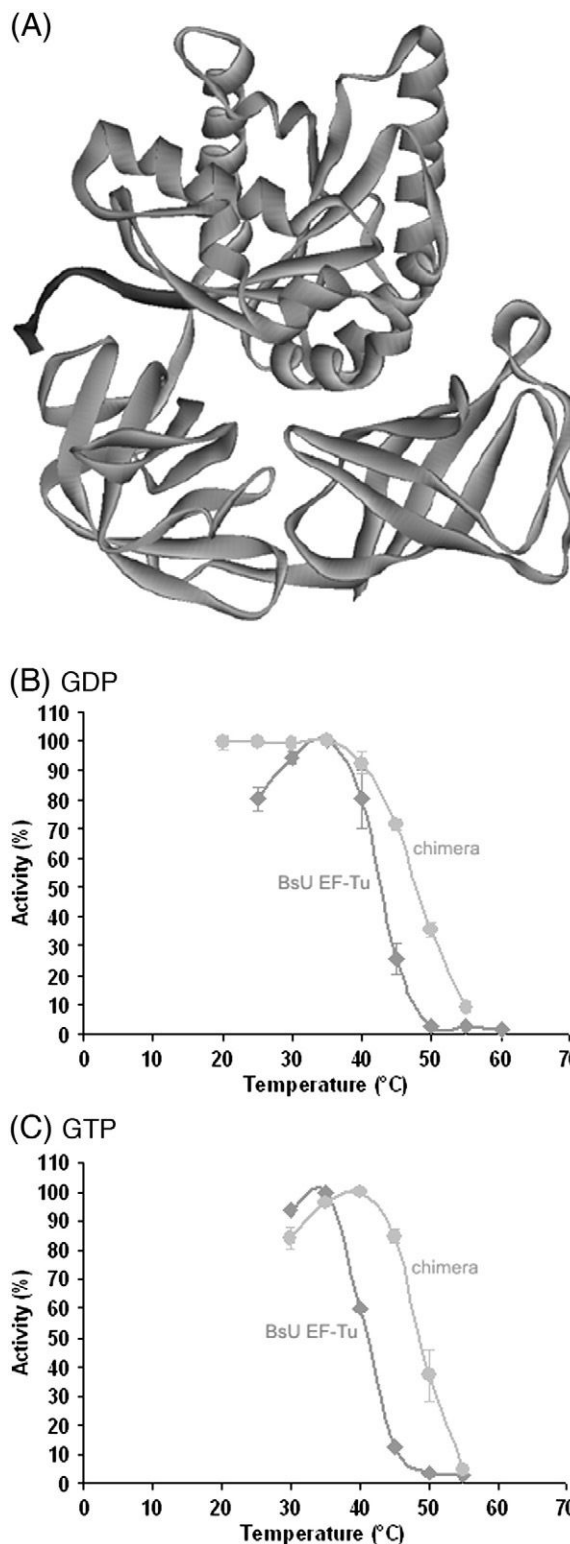**Fig. 7.** Effect of the N-terminal region in the context of three-domain EF-Tu. (A) The position of the N-terminal region is shown in the homology model of 3D structure of *B. stearothermophilus* EF-Tu in GTP form. Heat inactivation profiles of (B) GDP and (C) GTP forms of *B. subtilis* EF-Tu (gray diamonds) and *B. subtilis* EF-Tu chimeric protein containing *B. stearothermophilus* N-terminal region (light gray circles).

reciprocal chimera (1U-2U-3S-4S) displayed an opposite trend: the N-terminal region and the effector from *B. subtilis* destabilized the *B. stearothermophilus* G-domain (1U-2U-3S-4S) by about 5.8 °C and 11 °C in GDP and GTP conformations, respectively (Table 2B, Fig. 6). However, this destabilization was less pronounced than the destabilization by the N-terminal region alone (1U-2S-3S-4S). This result was consistent with the idea that the N-terminal and effector region from one organism are optimized to act together. Therefore, the N-terminal and effector region from *B. subtilis* destabilize the *B. stearothermophilus* G-domain less than does the *B. subtilis* N-terminal region alone.

We also conducted MD simulations (data not shown) and the results suggested that N-terminal regions from *B. subtilis* and *B. stearothermophilus* G-domains can adopt conformations leading to interactions with the effector region.

We concluded that the N-terminal and effector regions of the *B. stearothermophilus* G-domain may cooperate and that this cooperation may be important, at least in part, for the stability of the domain.

3.8. N-terminal region in the context of three-domain EF-Tu

As the main subject of this work was the single G-domain, it was important to validate the obtained results in the context of the whole three-domain molecule.

Therefore, the effect of the N-terminal region was evaluated within the three-domain EF-Tu from *B. subtilis*. A chimeric *B. subtilis* EF-Tu containing the N-terminal region from *B. stearothermophilus* EF-Tu (Fig. 7A) was prepared and examined.

Table 2C and Fig. 7B, C show that the thermostability of this chimeric *B. subtilis* EF-Tu was increased in comparison with that of the native *B. subtilis* EF-Tu by about 5.2 °C and 7.3 °C in GDP and GTP conformations, respectively. The less pronounced effect of the N-terminal region within the context of the three-domain molecule was expected since the full-length EF-Tu is about 100% larger than the G-domain alone and domains 2 + 3 provide additional thermostabilization (Fig. 1A).

These data showed that the N-terminal region appears to play an important role in the thermostability of both the isolated G-domain and also the full-length EF-Tu.

4. Concluding remarks

Previous low resolution studies identified the G-domain as key for EF-Tu thermostability. Here, a detailed systematic analysis of the G-domain demonstrated that a small portion of the *B. stearothermophilus* G-domain, the very N-terminal region, has a large impact on its thermostability, and this effect is significant also in the context of the three-domain EF-Tu.

Analogs of the N-terminal region of EF-Tu can be found in some other G proteins where they play an important role. An example is ADP-Ribosylation Factor-1 (ARF1). When ARF1 switches to the GTP state, its N-terminal residues may insert into membrane lipids [32]. In the ARF1/GDP crystal structure, the N-terminal residues 2–13 form a helix with its hydrophobic residues close to the effector region [33,34] in a way reminiscent of N-terminal–effector region interactions proposed here for EF-Tu.

The importance of a protein's N-terminal region for its thermostability has been described for some other proteins, such as GH11 xylanase, starch-branching enzyme, and flap endonuclease-1 [35–37]. It would be interesting to determine whether the N-terminal regions of additional G proteins other than EF-Tu may affect their thermostability.

Acknowledgements

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Paper 5

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The surface-associated elongation factor Tu is concealed for antibody binding on viable pneumococci and meningococci

Jan Kolberg[1], Sven Hammerschmidt[2], Ronald Frank[3], Jiří Jonák[4,5,6], Hana Šanderová[4,5] & Audun Aase[1]

[1]Department of Bacteriology and Immunology, Norwegian Institute of Public Health, Oslo, Norway; [2]Max von Pettenkofer Institut, Ludwig-Maximilians University of München, München, Germany; [3]Department of Chemical Biology, Helmholtz Centre for Infection Research, Braunschweig, Germany; [4]Department of Bacteriology, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic; [5]Laboratory of Molecular Genetics of Bacteria, Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic; and [6]Institute of Medical Biochemistry, First Medical Faculty, Charles University, Prague, Czech Republic

Abstract

Proteome analyses revealed that elongation factor-Tu (EF-Tu) is associated with cytoplasmic membranes of Gram-positive bacteria and outer membranes of Gram-negative bacteria. It is still debatable whether EF-Tu is located on the external side or the internal side of the membranes. Here, we have generated two new monoclonal antibodies (mAbs) and polyclonal rabbit antibodies against pneumococcal EF-Tu. These antibodies were used to investigate the amount of surface-exposed EF-Tu on viable bacteria using a flow cytometric analysis. The control antibodies recognizing the pneumococcal surface protein A and phosphorylcholine showed a significant binding to viable pneumococci. In contrast, anti-EF-Tu antibodies did not recognize pneumococcal EF-Tu. However, heat killing of pneumococci lacking capsular polysaccharides resulted in specific antibody binding to EF-Tu and, moreover, increased the exposure of recognized phosphorylcholine epitopes. Similarly, our EF-Tu-specific antibodies did not recognize EF-Tu of viable *Neisseria meningitidis*. However, pretreatment of meningococci with ethanol resulted in specific antibody binding to EF-Tu on outer membranes. Importantly, these treatments did not destroy the membrane integrity as analysed with control mAbs directed against cytoplasmic proteins. In conclusion, our flow cytrometric assays emphasize the importance of using viable bacteria and not heat-killed or ethanol-treated bacteria for surface-localization experiments of proteins, because these treatments modulate the cytoplasmic and outer membranes of bacteria and the binding results may not reflect the situation under physiological conditions.

Introduction

The Gram-positive *Streptococcus pneumoniae* and the Gram-negative *Neisseria meningitidis* are microorganisms that exclusively infect humans and colonize the nasopharynx. Bacterial surface structures are essential for interactions with receptors on host cells. The availability of complete genome sequences of these pathogenic microorganisms and recent improvements in high-resolution two-dimensional gel electrophoresis (2-DE) and peptide spot identification by MS have provided new insights into membrane-associated proteins. These proteins are of interest in order to understand bacterial virulence, to identify new candidates for vaccine development and to provide new targets for antimicrobial agents. 2-DE membrane analyses and immunochemical techniques indicated that cytoplasmic proteins such as the glycolytic enzymes enolase and glyceraldehyde-3-phosphate dehydrogenase are surface exposed in *N. meningitidis* (Ferrari *et al.*, 2006), *Listeria monocytogenes* (Schaumburg *et al.*, 2004) and *S. pneumoniae* (Bergmann *et al.*, 2001, 2004). The finding that these two proteins, besides their essential enzymatic activities, also bind plasminogen suggests a relevant physiological and pathogenic role for their surface localization.

Elongation factor-Tu (EF-Tu) is a further cytoplasmic protein that has recently been reported to be surface-associated

in *Streptococcus pyogenes* (Rodriguez-Ortega *et al.*, 2006; Severin *et al.*, 2007) and *N. meningitidis* (Ferrari *et al.*, 2006; Vipond *et al.*, 2006; Williams *et al.*, 2007). EF-Tu is involved in polypeptide elongation during protein synthesis and may thus represent a promising target for the development of antimicrobials (see Hogg *et al.*, 2002 for a review). EF-Tu is of one of the most abundant proteins in prokaryotes, representing about 5–10% of the total amount of proteins in *Escherichia coli* (Jacobson & Rosenbusch, 1976; Bosch *et al.*, 1983; Krab & Parmeggiani, 2002). This high cytoplasmic content necessarily raises the question as to whether detection of EF-Tu in bacterial membrane preparations might represent a contamination by cytosolic EF-Tu during their preparation. These effects can be circumvented in binding assays with antigen-specific antibodies and viable bacteria using a flow cytometric approach (Kolberg *et al.*, 2006). However, in some studies, bacterial inactivation techniques such as air drying or ethanol treatment have been used, raising the question as to whether a binding is a result of modified surface structures or not. We, therefore, aimed to use viable bacteria for flow cytometry, a technique that allows the analysis of surface accessibility of EF-Tu on a large number of bacteria. We have used polyclonal antibodies and monoclonal antibodies (mAbs) recognizing EF-Tu of pneumococci and meningococci. Here, we demonstrate that the surface exposition of EF-Tu on viable bacteria is not sufficient for recognition by our mAbs, and not even by the polyclonal antibodies. However, heating of pneumococci or ethanol treatment of meningococci exposed buried EF-Tu epitopes.

Materials and methods

Bacterial strains and culture conditions

Streptococcus pneumoniae strains ATCC 11733 (Spn 51, serotype 2), NCTC 10319 (Spn 37, serotype 35A), D39 (serotype 2) and its nonencapsulated derivative R6, the TIGR4 (serotype 4) and its nonencapsulated mutant FP23 were used in this study. The mutant FP23 was kindly provided by Francesco Iannelli, Siena, Italy (Pearce *et al.*, 2002). Construction of the *pspA* mutant used in this study was described previously (Kolberg *et al.*, 2006). Pneumococci were cultured in Todd–Hewitt broth (Roth, Karlsruhe, Germany) supplemented with 0.5% yeast extract (THY). *Neisseria meningitidis* group B clinical Norwegian isolate 44/76 (B:15: P1.7,16) and group B clinical New Zealand isolate NZ98/254 (B:4:P1.7b,4) were cultivated in Frantz' medium with shaking *E. coli* B, *Bacillus stearothermophilus* CCM 2184 and *Bacillus subtilis* 168 used for isolation of EF-Tu are described in Jonak (2007) and references therein. The other bacteria (besides *Lactococcus lactis* IL 1403) that were used in this study have been described previously (Kolberg *et al.*, 1997b).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Samples were subjected to SDS-PAGE with 4% stacking gel and 12% separating gel, electrotransferred to nitrocellulose membranes and probed with antibodies, as described previously (Kolberg *et al.*, 1997b).

Production of monoclonal antibodies

The procedure for mAb production and isotyping were performed as described (Kolberg & Sletten, 1996). Briefly, the supernatant from an overnight culture of the clinical Norwegian pneumococcal strain 1675/94 (serotype 12F) was heat-treated for 30 min at 60 °C and proteins were precipitated by adding solid ammonium sulphate to a final concentration of 60%. After centrifugation, the isolated proteins were extensively dialysed against water and then subjected to SDS-PAGE. Proteins in the range of 40–45 kDa were isolated by electroelution. The eluted proteins were dialysed against phosphate-buffered saline (PBS) and used for immunization of BALB/C mice. The subsequent fusion of spleen cells with NSO myeloma cells resulted in mAb 234,D-11. A protein fraction from the clinical Norwegian pneumococcal strain 1679/94 (7F) was used as an antigen for the production of mAb 244,G-2 (Kolberg *et al.*, 2006). To produce large amounts of antibodies, the hybridoma cell line 244,G-2 was cultivated in the Integra Cl 1000 chamber (Integra Biosciences AG, Switzerland). The mAb 244,G-2 was purified by affinity chromatography with protein A–Sepharose and for protein purification the antibody was coupled by a standard procedure to CNBr-Sepharose.

A high molecular mass protein fraction isolated by preparative SDS-PAGE electrophoresis from deoxycholate-extracted outer membrane vesicles (OMVs) from meningococcal strain 44/76 was used as an antigen for mAb 243,A-3. The protein reacting with this mAb was identified as heat shock protein 60 (Hsp60) after isolation of the protein by immunoaffinity chromatography, followed by N-terminal amino acid sequencing. The mAb 235,A-4 reacting with the meningococcal outer membrane protein, PorA, was obtained after immunization with LiCl/LiAc-extracted OMVs from the clinical isolate 394/98 from New Zealand. Whole-cell enzyme-linked immunosorbent assay (ELISA) and immunoblotting were used for specificity analyses of the PorA-specific mAb (Rosenqvist *et al.*, 1995).

The mAbs used in this study are listed in Table 1.

Deoxycholate extraction of pneumococcal proteins

The nonencapsulated *S. pneumoniae* R6 bacteria obtained after overnight growth in Todd–Hewitt broth were heat

treated for 40 min at 60 °C. The bacteria were washed once in PBS, followed by treatment for 10 min at 37 °C with mutanolysin (50 U mL^{-1}) and lysozyme (0.2 mg mL^{-1}). Bacterial cell walls were disrupted by several sonication cycles with cooling on ice between pulses. The suspension was centrifuged for 20 min at 12 000 g, 4 °C, and the pellet was extracted twice with 0.5% deoxycholate in 0.1 M Tris/HCl, pH 8.5, for 30 min.

EF-Tu proteins and their G-domains

To isolate pneumococcal EF-Tu, the deoxycholate extract described above was diluted to a detergent concentration of 0.1% and then loaded onto the mAb 244,G-2 affinity column (see Monoclonal antibodies above). After washing with 0.1% deoxycholate in 0.1 M Tris/HCl, pH 8.5, followed by buffer without deoxycholate, the proteins bound to the column were eluted with 4 M guanidine/HCl in PBS. After removal of the guanidine salt by dialysis against distilled water, eluted proteins were dissolved in 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate, 7 M urea, 2 M thiourea, 10 mM dithiothreitol, 40 mM Tris/HCl and 2.0% ampholyte pH 3–5. The BioRad Rotofor system was used for isoelectric focusing and the fractions containing proteins reacting with mAb 244,G-2 were identified by immunoblotting. For the final purification step, an SDS-PAGE with a 12% separating gel was used and electroelution was conducted.

    EF-Tu from *E. coli*, *B. stearothermophilus* and *B. subtilis* were prepared as described in Jonak (2007), and references therein. Recombinant G-domains of *E. coli* EF-Tu and *B. stearothermophilus* EF-Tu were prepared as described in Tomincova *et al.* (2002), Sanderova *et al.* (2004), and Jonak (2007).

Rabbit antibodies against pneumococcal EF-Tu

After electroelution of EF-Tu from the SDS-PAGE gel, the protein was extensively dialysed against PBS to remove SDS before being used as an immunizing antigen.

Pneumococcal EF-Tu peptide array and reactivity of the mAb

A membrane with 129 peptides consisting of 15 amino acids, each with an offset of three amino acids, was prepared as described (Frank, 2002). The measurement of mAb binding using a chemiluminescence substrate and Kodak Image Station 2000R was performed as described previously (Kolberg *et al.*, 2006).

MS

The purified mAb 244,G-2 reacting protein was subjected to in-gel trypsin digestion. Trypsin digests were analysed by matrix-assisted laser desorption ionisation (MALDI)

mass fingerprinting (Ultraflex; Bruker Daltonics, Bremen, Germany). Peptide masses were searched against the databases using the MASCOT program (Mascot, Matrix Science, London, UK) as described (Schaumburg *et al.*, 2004).

Isolation of pneumococcal cell-wall proteins

Pneumococci (R6) grown to the stationary phase were lysed using glass beads and the cell-wall fraction was isolated essentially as described (Bergmann *et al.*, 2001).

Meningococcal outer membranes

Deoxycholate-extracted OMVs were obtained from the Department of Biopharmaceutical Production, NIPH, Oslo. They were prepared as described (Fredriksen *et al.*, 1991).

Antibody binding to bacteria as analysed by flow cytometry

Pneumococci were grown to the log phase and binding of the different antibodies was measured as described (Kolberg *et al.*, 2006). Meningococci were cultivated under agitation in Frantz' medium. In binding experiments using killed bacteria, *S. pneumoniae* were heat inactivated at 56 °C for 30 min, whereas the *N. meningitidis* were killed by 70% ethanol for 1 h. The rabbit antibodies were used at a dilution of 1 : 100 and the mAbs were used as undiluted hybridoma cell culture supernatants. After incubation with primary antibody, unbound antibodies were washed away by two centrifugation cycles. Bound antibodies were detected by fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit immunoglobulins (Dako, Glostrup, Denmark) or FITC-conjugated sheep anti-mouse IgG (in-house prepared). The geometric mean fluorescence intensity (GMFI) of the various antibodies was recorded as a measure for binding activity.

Results and discussion

EF-Tu is recognized by mAb 234,D-11 and mAb 244,G-2

Immunizations of mice with detergent-extracted protein fractions from *S. pneumoniae* and subsequent fusions of spleen cells with NSO myeloma cells gave rise to mAbs, which, upon immunoblotting, reacted with a 43-kDa protein found in all examined pneumococcal strains.

    To identify the 43-kDa protein reacting with mAbs 234,D-11 (IgG2b) and 244,G-2 (IgG1) we performed immunoaffinty chromatography with mAb 244,G-2 bound to CNBr-Sepharose. Pneumococcal deoxycholate extracts were applied and eluted proteins were subjected to isoelectric focusing using the Rotofor system from BioRad. Fractions containing the 43-kDa protein were identified by

Table 1. Monoclonal antibodies used in this study

mAb	Isotype	Specificity	References
234,D-11	IgG2b	EF-Tu	This work
244,G-2	IgG1	EF-Tu	This work
230,B-9	IgG1	Streptococcal Hsp70	Kolberg et al. (2000)
145,F-2	IgM	Phosphorylcholine	Kolberg et al. (1997a)
149,B-3	IgG2a	PspA	Kolberg et al. (2001)
159,D-7	IgG2a	PspA	Kolberg et al. (2003)
243,A-3	IgG1	Meningococcal Hsp60	This work
151,F-9	IgG2b	PorA P1.16	Michaelsen et al. (2004)
235,A-4	IgG1	PorA P1.4	This work
144,H-3	IgG2a	Eubacterial ribosomal protein L7/L12	Kolberg et al. (1997b)

immunoblotting using our specific antibodies and then subjected to preparative SDS-PAGE. The separated proteins were electrotransferred to a polyvinylidene difluoride membrane. A strip with the 43-kDa protein was cut off for aminoterminal peptide sequencing. However, this strategy was unsuccessful and we were not able to obtain N-terminal sequences. Thus, we subjected the 43-kDa protein to MALDI mass-fingerprinting analysis. The mascot search showed that 23 out of 39 searched peptides matched to *S. pneumoniae* EF-Tu. The findings that we were unable to obtain N-terminal amino acid sequences from *S. pneumoniae* and *N. meningitidis* EF-Tu, the latter isolated from a 234,D-11 affinity column, suggest that the N-terminus in these proteins is blocked. This has also been observed in another study in which the N-terminal sequencing of EF-Tu from *Neisseria gonorrhoeae* was unsuccessful (Judd & Porcella, 1993).

The two mAbs recognize different EF-Tu epitopes

The EF-Tu molecule structure is highly conserved among bacteria. To confirm that our antibodies bind to EF-Tu and also recognize EF-Tu from other bacterial species, purified EF-Tu from *E. coli*, *B. stearothermophilus* and *B. subtilis* were used in immunoblot analysis. The results indicated reactivity with all bacteria and full-length EF-Tu proteins that were tested (Fig. 1). mAb 244,G-2 reacted with all four streptococcal species and the closely related *Enterococcus faecalis* and *L. lactis*. In contrast, this mAb reacted only with one of the two examined *Bacillus* species, namely *B. subtilis* (Fig. 1 and Table 2). The epitope was also expressed in the Gram-negative bacteria *Haemophilus influenzae* and *E. coli*. mAb 234,D-11 reacted only with two strains of the four examined streptococcal species and with the Gram-negative *N. meningitidis*. The finding of cross-reacting epitopes among some distant related genera, but not all, might indicate that the epitopes for these two mAbs are located in highly variable regions of the bacterial EF-Tu.
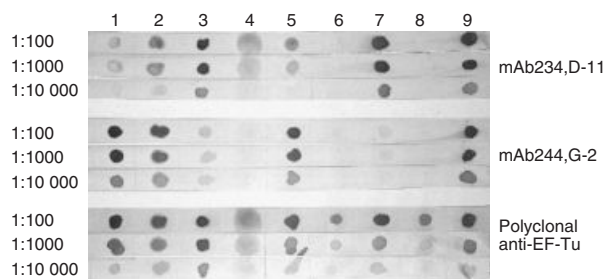

Fig. 1. Dot-blotting analysis under nondenaturing conditions using different dilutions of anti-EF-Tu specific mAbs and polyclonal rabbit antibodies raised against conventionally purified EF-Tu from *Streptococcus pneumoniae*. Lane 1, heat-treated whole pneumococcal strain R6 bacteria; lane 2, cell-wall fraction of strain R6; lane 3, ethanol-treated meningococcal strain NZ98/254, lane 4, OMVs NZ98/254; lane 5: *Escherichia coli* full-length EF-Tu; lane 6, domain 1 of EF-Tu from *E. coli*; lane 7: *Bacillus stearothermophilus* full-length EF-Tu; lane 8, domain 1 of EF-Tu from *B. stearothermophilus*; lane 9, *Bacillus subtilis* full-length EF-Tu. Ten micrograms of total protein from bacteria, 5 μg outer membrane proteins or 1 μg EF-Tu proteins were spotted on the membranes.

Table 2. Immunoblot reactivities of EF-Tu specific mAbs against bacterial whole-cell lysates

Organisms	mAb 234,D-11	mAb 244,G-2
S. pneumoniae	+	+
S. pyogenes	−	+
S. agalactiae	+	+
S. sanguis	−	+
E. faecalis	+	+
L. monocytogenes	−	+
L. lactis	+	+
B. stearothermophilus	+	−
B. subtilis	+	+
H. influenzae	−	+
N. meningitidis	+	−
E. coli	−	+

Surprisingly, mAb 234,D-11 shows relatively weak reactions with *S. pneumoniae* whole cells, but strong reactions with other bacteria (Fig. 1). One may speculate whether the mouse B cell that fused with the NSO myeloma cell and gave rise to hybridoma cell-line 234,D-11 was triggered by the immunizing *S. pneumoniae* proteins or rather that it had been induced/stimulated by microorganisms in the mouse.

The mAb epitopes are most likely located within EF-Tu domain 2+3

EF-Tu is a guanine nucleotide-binding protein that plays a central role in protein synthesis. EF-Tu is composed of three domains and the N-terminally located domain 1 contains the guanine nucleotide-binding site and is also called the catalytic or the G-domain (for a review, see Sanderova et al., 2004; Sanderova & Jonak, 2005; Jonak, 2007 and references

therein). Domain 2 and domain 3 are noncatalytic domains found to modulate properties and activities of domain 1. The mAb 244,G-2 reacted with full-length EF-Tu from *E. coli* but not with an isolated domain 1 under nondenaturing conditions in protein dot blot assays (Fig. 1) and under denaturing conditions by immunoblotting after SDS-PAGE (results not shown). This suggests that the epitope recognized by mAb 244,G-2 is located within domain 2 and/or domain 3 of the EF-Tu molecule. As expected from immunoblots with whole-cell lysates of *B. stearothermophilus*, our mAb 244,G-2 did not react with full-length *B. stearothermophilus* EF-Tu. In contrast, the mAb 234,D-11 recognized the EF-Tu of whole bacterial cell lysates and the EF-Tu from *B. stearothermophilus*. This mAb did not react with the isolated domain 1 of EF-Tu from *B. stearothermophilus*, indicating that the epitope for mAb 234,D-11 is most likely also recognizing an epitope within domain 2 and/or domain 3 of EF-Tu (Fig. 1 lane 8). At higher mAb concentrations, some cross-reactions with *E. coli* were seen (Fig. 1). Our control polyclonal anti-EF-Tu antibodies, which were raised against conventionally purified pneumococcal EF-Tu, reacted with both full-length proteins and the isolated domain 1 of EF-Tu (Fig. 1).

Further attempts to localize the epitopes were made using a peptide spot membrane. The 398 amino acid residues of the pneumococcal strain R6 EF-Tu were divided into 129 peptides with a length of 15 amino acid residues and an overlap of 12 amino acids. The same three adjacent peptides reacted with both mAbs, but also with a negative control mAb 151,F-9 (IgG2b), directed against the P1.16 epitope of PorA, an outer membrane protein on *N. meningitidis* (data not shown). In addition, mAb 234,D-11 bound to two adjacent peptides in a highly variable part in the N-terminal region of EF-Tu (data not shown). However, this result did not show any correlation to our positive immunoblot reaction patterns and common amino acid residues in the bacterial species examined. These results and the fact that the recombinant domain 1 did not react with this mAb indicate an unspecific reaction. The finding that we could not define the mAb-binding sites in EF-Tu using short peptides suggests that the mAbs do not recognize linear epitopes in EF-Tu but rather conformation-dependent epitopes. This was unexpected as strong immunoblot reactions suggested continuous linear epitopes. One possible explanation could be that stripping of SDS from the EF-Tu proteins during electro-transfer from the gel to the membrane allows a partial renaturation. The structure of the three domains EF-Tu allows a series of conformational changes when they bind substrates (for a review, see Krab & Parmeggiani, 2002; Jonak, 2007). It may be unlikely that such a renaturation should constitute epitopes that are built up of residues from all three domains. It is worth mentioning here that an inconclusive epitope mapping was reported for an EF-Tu-

specific mAb, which, unlike the two mAbs described here, recognized EF-Tus of almost all organisms both in the bacterial and in the archaeal kingdoms (Baensch *et al.*, 1998).

EF-Tu is not accessible for antibody binding on viable pneumococci

Besides its role in protein synthesis, EF-Tu has been described to be cell surface associated in *Lactobacillus johnsonii*, thereby mediating attachment of this lactic acid bacteria to human intestinal cells and mucins and for inducing proinflammatory responses (Granato *et al.*, 2004). EF-Tu has also been shown to be part of the cell-wall subproteome of the facultative intracellular pathogen *L. monocytogenes* (Schaumburg *et al.*, 2004) and a membrane protein in *Bacillus anthracis* (Chitlaru *et al.*, 2007). The isolated pneumococcal cell wall proteins contained EF-Tu as shown by the reactivity with both mAbs and polyclonal antibodies against EF-Tu under neutral conditions in dot blots (Fig. 1). To determine whether EF-Tu is present on the external side of *S. pneumoniae* and accessible for antibody binding, flow cytometry experiments were performed with viable bacteria. Antibodies against the surface protein PspA (pneumococcal surface protein A) were used as positive controls (Kolberg *et al.*, 2006). The polyclonal rabbit anti-PspA antibodies reacted strongly with all examined strains (GMFI range 14–71), with the exception of the pneumococcal *pspA* mutant Spn51ΔpspA. The fluorescence intensities of the rabbit antibodies against EF-Tu did not provide a clear experimental evidence for surface localization of the EF-Tu protein (Table 3). As we assumed that the capsular polysaccharide might mask the EF-Tu as has been shown for other surface proteins including the PspA and PspC (Kolberg *et al.*, 2006), we also used capsule-deficient strains in our binding experiments. The effect of the capsule on antibody binding to EF-Tu and other surface proteins of viable pneumococci was analysed with the TIGR4 non-encapsulated isogenic mutant FP23 and the nonencapsulated strain R6. The R6 is a derivative of the encapsulated serotype 2 strain D39. Using the rabbit-derived polyclonal anti-EF-Tu antibodies, our flow cytometry data indicated a very faint binding to FP23 and R6 (both GMFI 1.1) compared with the encapsulated strains TIGR4 and D39 (both GMFI 0.6) (Table 3).

The anti-EF-Tu specific mAbs 234,D-11 and 244,G-2 did not bind to encapsulated and nonencapsulated pneumococci (Table 3) whereas the PspA specific mAbs 149,B-3 and 159,D-7 showed the surface accessibility of the recognized PspA epitopes in some of the strains (Table 3). The intensity of binding of the mAbs and polyclonal antibodies against PspA depends on the PspA type and the amount of capsular polysaccharide of the strains used. PspA is an antigenically

Table 3. Flow cytometric measurements of antibody binding to pneumococci*

Strain	Rabbit anti-EF-Tu	Preimmune serum	mAb 234,D-11 EF-Tu	mAb 244,G-2 EF-Tu	Rabbit anti-PspA	mAb 149,B-3 PspA	mAb 159,D-7 PspA	mAb 145,F-2 PC	mAb 230,B-9 Hsp70
TIGR4									
Live	0.6	0.5	0.7	0.7	19	15	0.6	2.9	0.6
Dead	0.9	0.7	0.9	1.0	11	10	0.7	7.3	0.7
FP23									
Live	1.1	0.8	0.8	1.0	28	41	0.6	34	0.7
Dead	23	1.9	4.1	17	35	35	1.0	51	2.2
D39									
Live	0.6	0.6	0.6	0.7	14	0.6	1.6	1.1	0.7
Dead	0.9	0.7	0.7	0.7	14	0.6	1.2	2.4	0.7
R6									
Live	1.1	0.9	1.0	0.9	28	0.8	5.9	27	0.9
Dead	6.8	1.4	2.6	6.5	52	0.9	13	69	1.1
Spn 51									
Live	0.9	0.8	0.7	0.9	46	2.4	8.6	13	0.6
Dead	3.8	0.8	1.4	2.5	34	0.8	7.6	9.3	0.8
Spn 51 PspA-									
Live	0.9	0.9	0.7	0.7	0.5	0.6	0.6	16	0.7
Dead	1.3	1.2	1.6	1.3	0.7	0.9	0.9	57	0.9
Spn 37									
Live	0.7	0.8	0.8	0.9	71	0.7	26	11	0.7
Dead	2.1	0.9	1.0	1.7	35	0.7	17	22	0.8
1675/94									
Live	0.6	0.5	0.6	0.7	39	0.6	11	3.5	0.7
Dead	0.6	0.5	0.6	0.7	26	0.7	8.5	3.5	0.6

*Results given as geometric mean fluorescence intensity (GMFI).

highly variable protein and has been divided into two major families (Hollingshead *et al.*, 2000). The mAb 149,B-3 reacted only with TIGR4 belonging to PspA family 2 (Shaper *et al.*, 2004). In contrast, mAb 159,D-7 did not react with TIGR4, but strongly with strains Spn 37 and Spn 51, and moderately to weakly with some of the other examined strains. Similar to earlier results (Kolberg *et al.*, 2006), the capsular polysaccharide masks the PspA and encapsulated strains showed a significantly reduced fluorescence intensity compared with their nonencapsulated mutants. This is in contrast to flow cytometric findings by Daniels *et al.* (2006) who found that the capsular polysaccharides of type 2 and 3 pneumococci showed little or no ability of inhibiting the two PspA specific mAbs used in their studies. Reasonable explanations could be different epitope locations of the mAbs used in these studies and their studies or use of strains of different capsular types. However, D39 (serotype 2) was used in both studies. It should be noted that the binding of our polyclonal rabbit antibodies against PspA was also masked by capsular polysaccharides (Table 3). The binding of the phosphorycholine-specific mAb 145,F-2 was largely, but not completely, blocked by capsular polysaccharides (Table 3). This result is in line with a recent study (Daniels *et al.*, 2006). The mAb 230,B-9, which was used as a control antibody and detects an epitope of the pneumococcal

Hsp70, was nonreactive in accordance with its cytoplamic localization and our previous data (Kolberg *et al.*, 2000, 2001, 2003).

Exposure of EF-Tu in nonencapsulated pneumococci after heat treatment

In order to investigate whether modifications of the pneumococcal cell wall surface influence the surface localization of pneumococcal EF-Tu or known pneumococcal surface components, the bacteria were heat treated for 30 min at 56 °C. The flow cytometric analysis indicated a strong increase in the fluorescence intensity and hence an increased binding of the rabbit antibodies and the mAbs recognizing EF-Tu when the nonencapsulated TIGR4 (serotype 4) mutant FP23 and the nonencapsulated R6 were tested (Table 3). A moderate increase in fluorescence intensity was measured for Spn 51 (serotype 2) (Table 3). Binding of the anti-PspA antibodies, polyclonal and mAb, was increased in some pneumococcal strains but decreased in other pneumococcal strains (Table 3). More importantly, the heat treatment and reaction with anti-PspA antibodies did not change nonreacting strains into positively reacting strains. Regarding the reaction with antiphophorycholine antibodies, with the exception of strains Spn 51 and 1675/94, the

use of killed pneumococci resulted in an increase in the fluorescence intensity for the phosphorylcholine-specific mAb 145,F-2 (the relative fold increase was in the range 1.5–3.6-fold, GMFI dead bacteria vs. GMFI live bacteria). Heat killing caused only minor changes in the binding of the anti-Hsp70-specific mAb 230,B-9, indicating that the membrane integrity has not been destroyed by the heat treatment.

EF-Tu is not accessible for antibody binding on viable meningococci

OMVs from *N. meningitidis* are used as vaccines against serogroup B meningococcal diseases. They can be prepared by extraction with the detergent deoxycholate (Fredriksen *et al.*, 1991). In addition to extract vesicles, the deoxycholate also removes lipopolysaccharides (endotoxin) that are responsible for acute inflammatory responses and tissue destruction characteristic of meningococcal infection (Brandtzaeg *et al.*, 1992). A detailed knowledge of the OMV composition is essential for the understanding of the immune responses of the vaccines and for standardization of the manufacturing process. Among the strains that are used for vaccine production are the Norwegian strain 44/76 and the New Zealand strain NZ98/254. Proteome data of OMV preparations have shown that EF-Tu, among some other cytoplasmic proteins, is membrane associated (Ferrari *et al.*, 2006; Vipond *et al.*, 2006; Williams *et al.*, 2007). Our dot-blot assay using OMVs from strain NZ98/254 showed weak reactions with mAb 234,D-11 and rabbit antibodies raised against purified pneumococcal EF-Tu (Fig. 1). The cross-reactivity of the polyclonal anti-EF-Tu antibodies was expected as EF-Tu is a highly conserved protein. To assess whether EF-Tu is associated with the external side of the meningococcal outer membrane, we performed flow cytometric analysis with viable meningococcal strains 44/76 and NZ98/254. Neither the polyclonal rabbit antibodies nor the mAbs against EF-Tu showed positive shifts in the fluorescence intensity whereas the mAbs against the highly variable outer membrane protein PorA showed the expected increase in fluorescence intensity (Table 4). The mAb 151,F-9, which is directed against the PorA, P1.16 epitope in strain 44/76,

but cannot recognize the PorA in strain NZ98/254, reacted as expected only with strain *N. meningitidis* 44/76. In contrast, the mAb 235,A-4, which is directed against the P1.4 epitope on PorA of strain NZ98/254, bound only to this strain. In accordance with previous experiments, our control mAb 144,H-3, directed against an epitope on the cytoplasmic ribosomal protein L7/L12, did not bind to the two examined meningococcal strains (Michaelsen *et al.*, 2001). Similar results were found for mAb 243,A-3, reacting with the meningococcal Hsp60. This mAb was prepared using a protein fraction from OMVs of strain 44/6 and Hsp60 is thus a membrane-associated protein as has been shown by others using 2-DE (Ferrari *et al.*, 2006; Vipond *et al.*, 2006; Williams *et al.*, 2007), but the region carrying the epitope is most likely concealed in viable meningococci.

Ethanol treatment of meningococci makes EF-Tu available for antibody binding to some extent

Unlike Gram-positive pneumococci, the Gram-negative meningococci were not sufficiently killed by our heating procedure (30 min, 56 °C). Therefore, we treated meningococci with 70% ethanol for 30 min at room temperature. Using the rabbit anti-EF-Tu antibodies, the ethanol treatment caused a relative ninefold and 23-fold increase in GMFI dead bacteria vs. viable bacteria for the *N. menigitidis* strains NZ 98/254 and 44/76, respectively (Table 4). The increase in GMFI for mAb 234,D-11 was also slightly higher compared with that measured for the other antibodies after ethanol treatment of meningococci (Table 4). This may indicate that EF-Tu of meningococci is located in the meningococcal outer membrane similar to the EF-Tu of pneumococci. This is very similar to what we have observed for some PorB epitopes on group B meningococci (Michaelsen *et al.*, 2001). In a previous study, using a similar flow cytometry method, the meningococcal EF-Tu was reported to be surface exposed in group B meningococci (strain MC58) (Ferrari *et al.*, 2006). However, in this study, only ethanol-killed bacteria were tested, and they concluded that EF-Tu was associated with the external side of the bacterial membrane. Our findings demonstrate that EF-Tu is not directly available for antibody binding on viable

Table 4. Flow cytometric measurements of antibody binding to meningococci*

Strain	Rabbit anti-EF-Tu	Preimmune serum	mAb 234,D-11 EF-Tu	mAb 244,G-2 EF-Tu	mAb 151,F-9 PorA	mAb 235,A-4 PorA	mAb 243,A-3 Hsp60	mAb 144,H-3 ribosomes
NZ 98/254								
Live	0.7	0.6	0.6	0.7	1.7	9.7	0.6	0.6
Dead	6.5	3.4	4.9	1.5	2.1	28	3.7	3.3
44/76								
Live	0.6	0.5	0.7	0.7	23	0.7	0.7	0.6
Dead	14	5.2	10	1.9	24	1.6	7.3	5.6

*Results given as geometric mean fluorescence intensity (GMFI).

meningococci and thus is most probably concealed below other meningococcal surface structures.

Concluding remarks

Our flow cytometric analyses revealed no binding of both polyclonal rabbit antibodies and mAbs against EF-Tu on log phase-grown viable pneumococci and meningococci. In contrast, control antibodies recognizing major surface proteins on these bacteria showed high reactivities whereas mAbs against cytoplasmic structures such as ribosomal proteins were nonreactive.

Both meningococci and pneumococci are highly pathogenic organisms. They are therefore often killed by various methods before examinations in *in vitro* assays. Our results indicate that heat treatment modulates the pneumococcal cytoplasmic membranes in such a way that surface-associated EF-Tu is made accessible for binding of antibodies. However, this is found only for the two examined nonencapsulated strains and thus, antibody binding depends on the amount of capsule expression. Killing of *N. meningitidis* group B strains by ethanol treatment showed a substantial increase in the binding of mAb 234,D–11 and rabbit polyclonal antibodies against EF-Tu. Taken together, our results indicate that EF-Tu seems to be buried in the external membranes on viable pneumococci and meningococci, and is thus not available for antibody binding. However, we have to consider that the lack of EF-Tu accessibility on viable bacteria as seen under the experimental conditions used here might not necessarily reflect the *in vivo* situation.

Acknowledgements

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