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Review

Bacterial elongation factors EF-Tu, their mutants, chimeric forms, and domains: Isolation and purification☆

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Abstract

Prokaryotic elongation factors EF-Tu form a family of homologous, three-domain molecular switches catalyzing the binding of aminoacyl-tRNAs to ribosomes during the process of mRNA translation. They are GTP-binding proteins, or GTPases. Binding of GTP or GDP regulates their conformation and thus their activity. Because of their particular structure and regulation, various activities (also outside of the translation system) and a relative abundance they represent attractive tools for studies of many basic but still not fully understood mechanisms both of the translation process, the structure–function relationships in EF-Tu molecules themselves and proteins and energy transduction mechanisms in general. The review critically summarizes procedures for the isolation and purification of native and engineered eubacterial elongation factors EF-Tu and their mutants on a large as well as small scale. Current protocols for the purification of both native and polyHis-tagged or glutathione-*S*-transferase (GST)-tagged EF-Tu proteins and their variants using conventional procedures and the Ni-NTA-Agarose or Glutathione Sepharose are presented.

Keywords: Bacterial elongation factors EF-Tu; Detection methods; Separation methods; Purification methods; EF-Tu.GDP; EF-Tu.EF-Ts; EF-Tu mutants; G-domains; Protein synthesis; *Escherichia coli*; *Bacillus stearothermophilus*; *Bacillus subtilis*; *Streptomyces aureofaciens*; *Streptomyces coelicolor*; *Thermus aquaticus*; *Thermus thermophilus*

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Abbreviations: EF-Tu, EF-Ts and EF-G, prokaryotic elongations factors Tu, Ts and G, respectively; EF-1α, eukaryotic elongation factor 1α; GDPCP, guanosine 5′-(β,γ-methylenetriphosphate); GDPNP, guanosine 5′-(β,γ-imidotriphosphate); ppGpp, 5′-diphosphate 3′-diphosphate; Ni-NTA-agarose, Ni^{2+}-nitrilotriacetic acid agarose; GST, glutathione-*S*-transferase; SDS, sodium dodecyl sulfate; TPCK, *N*-tosyl-L-phenylalanylchloromethane; Ec, *Escherichia coli*; Bst, *Bacillus stearothermophilus*

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1. Introduction

The first step in the process of peptide chain elongation on ribosomes is the binding of codon-specified aminoacyl-tRNA to the ribosomal aminoacyl or A-site.

Proteins essential for this reaction have been found in both prokaryotic and eukaryotic organisms. The aminoacyl-tRNA binding processes are similar in these two types of organisms but the proteins are not interchangeable and differ in their properties. The eubacterial proteins are called elongation factors EF-Tu and the archaebacterial and eukaryotic proteins are called elongation factors EF-1α. The protein synthesis depends almost absolutely on these factors. This review describes procedures developed for the purification of eubacterial elongation factors EF-Tu.

Eubacterial EF-Tus are monomeric, medium size, moderately acidic proteins with a molecular mass ranging from 40 to 45 kDa and a theoretical p*I* around 5.0, depending on the bacterial species (*Escherichia coli* EF-Tu: 393 amino acid residues; 43 kDa; p*I* = 5.30). They belong to the superfamily of GTP-binding proteins. Therefore, they bind guanine nucleotides, GDP, GTP, and also ppGpp and display a slow intrinsic GTPase activity. EF-Tu further forms complexes with aminoacyl-tRNAs and with another elongation factor EF-Ts and, in addition, it interacts with some regions of the ribosome [1–3]. The reactions of aminoacyl-tRNA delivery to ribosomes promoted by EF-Tu are summarized in the following equations:

EF-Tu.GDP + EF-Ts + GTP
↔ EF-Tu.GTP + EF-Ts + GDP

aminoacyl-tRNA + EF-Tu.GTP
↔ EF-Tu.GTP.aminoacyl-tRNA

EF-Tu.GTP.aminoacyl-tRNA + ribosome.mRNA
→ aminoacyl-tRNA.ribosome.mRNA + EF-Tu.GDP + Pi

As determined by X-ray analysis of available crystalline complexes of EF-Tu, its molecule (Fig. 1) consists of three domains 1, 2 and 3 (numbering from the N-terminus) [4,5]. Their relative positions change dramatically upon exchanging GDP for GTP [6–9] that results in the formation of a binding pocket for aminoacyl-tRNA. Thus, conformational transitions induced by GDP and GTP regulate EF-Tu activity in an allosteric manner. The GDP/GTP binding and the GTPase center of EF-Tus is situated in the N-terminal domain of the protein. Thus, the domain 1 is also called the catalytic or G-domain [10]. Domains 2 + 3 (non-catalytic domains) were found to remarkably, and differently, depending on the bacterial species, modulate the properties and activities of domain 1 [10–12].

EF-Tu also forms one of the four subunits of RNA-phage replicase, acting in complex with EF-Ts in the initiation step and it was reported to directly interact with the C-terminal region of the β-subunit of the RNA polymerase [3].

Elongation factors EF-Tu represent a family of highly homologous components allowing studies of evolutionary relationships between prokaryotic organisms as well as the identification in protein molecules of structural features of adaptation to various living conditions. For many years EF-Tus also played a role of functional and structural model proteins for the whole family of GTP-binding proteins. Through application of protein engineering techniques elongation factors Tu became in the last about 20 years a popular target for studies of structure–function relations in protein molecules as well as of the significance of domain structure for protein functions.

2. Assay methods for EF-Tu

The capacity of EF-Tu to bind GDP with a high affinity (in the nanomolar range) and to exchange it for radiolabeled GDP has become the basis of the most reliable, simple and rapid assay for these proteins [13,14]. These analyses can be performed accurately due to EF-Tu.GDP complex ability to bind quantitatively to cellulose nitrate filter discs. Using tritium-labeled GDP, picomole quantities of EF-Tu GDP can be conveniently measured. In brief, 10–300 nM EF-Tu.GDP (as determined by Bradford or Lowry assay, see later) from *E. coli* (EcEF-Tu.GDP) is incubated in a binding buffer (50 mM Tris/HCl (pH 7.6), 10 mM $MgCl_2$, 60 mM NH_4Cl, 10 mM 2-mercaptoethanol) containing a saturating level (2–5 μM) of [^{3}H]GDP (commercial [^{3}H]GDP is usually diluted with cold GDP to the desired concentration) for 30 min at 0 °C, or at 37 °C, or at a temperature optimal for a particular EF-Tu. The reaction (final volume 50–100 μl) is stopped by dilution with 1–3 ml of the cold binding buffer and the mixture passed through a cellulose nitrate filter disc (Millipore HAWP02500, pore size 0.45 μm; Sartorius SM11306, 0.45 μm), the filter washed with 3 × 3 ml of the binding buffer to remove unbound GDP, dried and measured. EF-Tu appears to be the only protein, at least in *E. coli* and all other examined bacteria, which binds GDP tightly and is absorbed to the filter under these conditions. (Commercial preparations of GDP are often contaminated with GMP, GTP, ppGpp and/or other contaminants. A procedure for GDP purification is described in [15].)

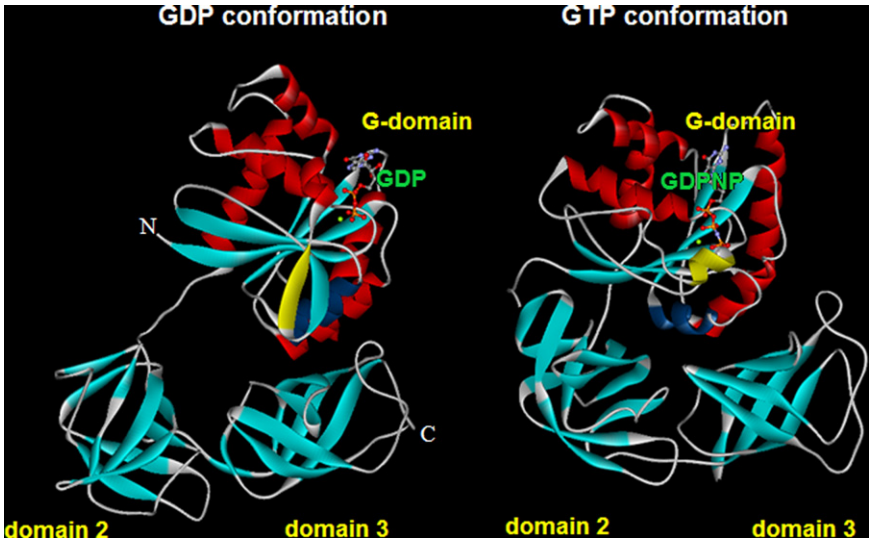

Fig. 1. Ribbon representation of the three-dimensional homology model of *Bacillus stearothermophilus* EF-Tu.GDP and EF-Tu.GDPNP.

The fact that under saturating conditions, GDP forms an equimolar complex with intact EF-Tu makes the filter-binding assay a suitable tool for the **determination of percentage of active molecules of EF-Tu and its variants in a preparation**. The percentage of active EF-Tu is determined from the point at which the factor was saturated with GDP (after correcting for the background: retention of [^{3}H]GDP on a filter in a minus EF-Tu control). The 100% active protein should bind 1 mol of GDP per 1 mol of EF-Tu.

The **concentration of EF-Tu proteins** in a preparation is most frequently determined by the Bradford procedure [16] or by the Lowry procedure [17] standardized against bovine serum albumin. At present, apparently the most precise EF-Tu protein quantification can be carried out by quantitative amino acid analysis; according to our experience the values obtained are smaller than those obtained by the Bradford or Lowry procedures (H. Šanderová and J. Jonák, unpublished results). Another way to determine the EF-Tu concentration is by a spectroscopic measurement at 280 μm. The extinction coefficient ε_{280} of EF-Tu.GDP from *E. coli* was found to be 29200 M^{-1} cm^{-1} by quantitative amino acid analysis [18]. This extinction coefficient is, in contrast to some previous estimations of e.g. 41600 M^{-1} cm^{-1} [19], close to the theoretical value of 26600 M^{-1} cm^{-1}, which was determined by summing the molar extinction coefficients of the absorbing species (3 Cys, 1 Trp, 10 Tyr) present in EF-Tu and using a molar extinction coefficient for GDP at 280 nm of 7800 M^{-1} cm^{-1}.

The ability of EF-Tu factors to promote polypeptide synthesis on a ribosome–mRNA template in the presence of aminoacyl-tRNA and elongation factor EF-G or to bind aminoacyl-tRNA to ribosome in the presence of appropriate mRNA can also be used as assays for EF-Tu. In practice one usually measures polyphenylalanine synthesis directed by polyU or Phe-tRNA binding to the polyU-ribosome complex. However, these methods require a comparatively complex systems, are subject to variability in the ribosome preparations, and the results in units of phenylalanine polymerized or Phe-tRNA bound to the ribosome cannot be easily and directly translated into molar quantities of EF-Tu. Besides, Phe-tRNA binding to ribosomes in response to polyU takes place at 10–20 mM Mg^{2+} even without EF-Tu and a factor-free phenylalanine polymerization on the ribosomes, even though remarkably slow, has also been described and well characterized [20,21].

To conclude, at present, the (GDP binding) filter assay is practically the only one currently in use and generally accepted for the detection of EF-Tu and for the evaluation of its activity. However, it has some limitations originating from the nature of EF-Tu proteins and relationships between their functional sites. Generally speaking, if EF-Tu does not bind GDP, it is considered to be really "dead" and will certainly neither bind aminoacyl-tRNA nor promote amino acid polymerization. This is due to the fact that for the formation of the binding site for aminoacyl-tRNA on EF-Tu a transition in its conformation is absolutely required that can only be induced by binding of GTP [22,23]. On the other hand, the activity to bind GDP does not fully guarantee the ability of EF-Tu to bind aminoacyl-tRNA and to participate in the polymerization process. It is possible to selectively (by TPCK reagent) disturb these two latter functions of EF-Tu without significantly affecting the ability to bind GDP [24,25]. This is mainly due to the fact that the GDP/GTP binding site and aminoacyl-tRNA binding site are on EF-Tu molecule spatially separated [22,23].

3. Purification of EF-Tu

3.1. General comments

EF-Tu from *E. coli* and apparently many other (presumably non-thermophilic) microorganisms is a thermolabile protein ("u" in EF-Tu originally stays for unstable); 50% inactivation takes place after 8 min at 49–52 °C for EcEF-Tu.GDP and at 41–47 °C for EcEF-Tu.GTP [11,26]. In the absence of EF-Ts, GTP or GDP it becomes even less stable; at 4 °C the free EF-Tu loses its activity within a few hours [27]. The addition of at least

10% glycerol and the omission of magnesium ions were reported to be necessary to slow down the free EF-Tu denaturation. By an unknown mechanism, magnesium ions precipitate free but not GDP-bound EcEF-Tu [28]. It has practical consequences. To regenerate EF-Tu-guanine nucleotide complex back from nucleotide-free EF-Tu, the nucleotides (GDP, GTP, GDPCP, GDPNP, etc.) must be added first, before magnesium ions addition. Thus, inactivation of free EF-Tu can be stopped and perhaps also reversed in part by GDP + magnesium and this effect can be accelerated by kirromycin [3]. Or, in their absence, by EF-Ts. The nucleotide-free complex of EF-Tu with EF-Ts is stable [29]. In contrast, *Euglena gracilis* EF-Tu from chloroplasts was reported to be stable in the absence of guanine nucleotides [30]. Similarly, nucleotide-free EF-Tu from thermophilic *Thermus thermophilus* was found to be remarkably stable, too [31,32].

EF-Tu mutants and variants that have an impaired nucleotide binding are apparently not correctly folded and display a decreased solubility on overexpression in the *E. coli* cell. It was found that co-overexpression of EF-Ts at a 1:1 ratio with such mutant EF-Tu (e.g. from a vector that coexpresses glutathione-*S*-transferase-fused EF-Tu and EF-Ts) can dramatically improve the solubility of the mutant EF-Tu and increases the yield of the recombinant protein [33]. It appears that for the formation of the correct EF-Tu structure the nucleotide plays an important role as a "folding nucleus", and also that in its absence, or in case of a reduced nucleotide affinity, EF-Ts can act as a folding template or steric chaperone for the correct folding (and maximum solubility and long-term stability) of EF-Tu [3]. In the presence of GDP and magnesium ions, EF-Tu (*E. coli*) can be stored in the form of crystals, e.g. in 35–42% ammonium sulfate solution in 20 mM Tris–HCl (pH 7.6), 10 mM magnesium chloride, 5 mM mercaptoethanol, 10 μM GDP at 4 °C or in a soluble form (most of EF-Tus) in 10–50% glycerol in a similar buffer (without ammonium sulfate) at −20 °C, and at concentrations about 10 mg/ml or higher, for several years without any apparent loss of activity (J. Jonák, unpublished).

3.2. Purification procedures-an overview

Purification of EF-Tus (according to their origin)
Natural EF-Tus:

(i) conventional column chromatography (anion-exchanger and gel filtration)
(ii) affinity chromatography on GDP-Sepharose
(iii) affinity chromatography on Thiol-Sepharose
(iv) spontaneous aggregation

Untagged recombinant EF-Tus:

(i) heat treatment
(ii) column chromatography in the presence of kirromycin

Tagged recombinant EF-Tus:

(i) affinity chromatography on Ni-NTA-Agarose
(ii) affinity chromatography on Glutathione-Sepharose

Purification of recombinant G-domains

untagged G-domains: conventional column chromatography (anion exchanger and gel filtration)
GST-tagged G-domains: affinity chromatography on Glutathione-Sepharose

Purification of EF-Tus and their variants (according to scale)
Large scale procedures:

(i) conventional column chromatography
(ii) heat treatment
(iii) column chromatography in the presence of kirromycin
(iv) spontaneous aggregation

Small scale procedures:

(i) affinity chromatography on GDP-Sepharose
(ii) affinity chromatography on Thiol-Sepharose
(iii) affinity chromatography on Ni-NTA-Agarose
(iv) affinity chromatography on Glutathione-Sepharose.

3.2.1. Natural EF-Tus

EF-Tu is one of the most abundant bacterial proteins. It comprises as much as 5–10% of the cytoplasmic protein in *E. coli* [3] and in all bacteria investigated till now. Because of their abundance in the cell and solubility in aqueous media considerable amounts of EF-Tus can be isolated from bacteria by **conventional procedures** (column chromatography on an anion-exchanger followed by gel filtration chromatography). Similar purification schemes are usually applicable to EF-Tus from various bacterial sources. Tens of milligrams of purified wild-type EF-Tu can be prepared by this approach. The EF-Tu proteins prepared in this way can be sometimes a little heterogeneous because, in some bacteria, EF-Tu is encoded by two (e.g. in *E. coli*) to three (e.g. in *Streptomycetes*) unlinked genes [2]. Another source of the heterogeneity may be posttranslational modifications: phosphorylation [34–37], and methylation [38,39].

3.2.2. Cloned EF-Tus and G-domains

Cloning and overproduction of EF-Tu in a convenient host (usually in *E. coli*) may be a way to overcome these problems, however the purification procedure requires separation of the recombinant protein from large amounts of host-encoded EF-Tu. To be feasible, **cloned EF-Tu should posses some special features to separate it from host EF-Tu**.

For example, recombinant *Thermus thermophilus, Thermus aquaticus*, and *Thermotoga maritima* EF-Tus (all of thermophilic organisms) overproduced in *E. coli* cells, remain all **active after heating at 65 °C** when EcEF-Tu is completely denatured and precipitated and can be quantitatively removed with most other *E. coli* proteins simply by centrifugation [23,40–44]. Beside the purification of EF-Tu, Blank et al. [43] also described purification of EF-Ts and EF-G from *T. thermophilus* by this heat treatment.

Another approach was to overproduce the required (mutated) EF-Tu in *E. coli* strain PM 1455 producing its own EF-Tu kirromycin-resistant. This allows separation of the cloned EF-Tu from the host EF-Tu on the basis of different affinities for the antibiotic. The recombinant EF-Tu (it is expected to be naturally kirromycin-sensitive) forms **a complex with** added **kirromycin**, whereas the host kirromycin-resistant EF-Tu does not. Binding of kirromycin gives the recombinant EF-Tu an additional negative charge so that it is eluted from an anion-exchange column at a higher concentration of KCl than the resistant EF-Tu. To free the recombinant EF-Tu from bound kirromycin, about twice molar excess of EF-Ts over EF-Tu.kirromycin is added and the recombinant EF-Tu is separated in the form of EF-Tu.Ts from kirromycin by another anion-exchange chromatography (in the absence of Mg^{2+} ions and GDP) [45]. By this sophisticated but rather laborious procedure five mutants of EF-Tu proteins were successfully prepared (in mg quantities) and characterized [26,46–49]. Naturally, mutations in EF-Tu affecting the affinity for EF-Ts could compromise the EF-Tu.Ts step of the procedure [49]. Some mutations could also negatively interfere with the host translation apparatus leading to the appearance of revertants, inhibition of cell growth or formation of inclusion bodies.

Still different approaches to isolate EF-Tu were developed using the principle of **affinity chromatography**.

GDP-Sepharose was the first affinity carrier to show to isolate, in one step, a small amount of pure EF-Tu GDP from S-100 bacterial extracts [50] and the method is still in current use. The specificity of the method is based on the fact that EF-Tu appears to be the only bacterial protein binding GDP with high affinity (see above). A small DEAE-Sepharose/DEAE Sephadex A-50 column [51] or a MonoQ column (Pharmacia) were sometimes used as a final step to separate affinity isolated EF-Tu from nonspecific GTPase activities. The preparation of GDP-aminohexyl-Sepharose in the laboratory for EF-Tu.GDP isolation is described in [51], application of a commercial GDP-agarose is described in [52] and an example of versatility of this method is demonstrated in the case of purification of EF-Tu.GDP from the thermophilic hydrogen oxidizing *Calderobacterium hydrogenofylum* [53].

In another affinity method called "covalent" chromatography on **thiol-Sepharose**, EcEF-Tu and EcEF-G are purified in a single procedure on the basis of the presence of reactive accessible cysteines in their molecules. This allows their selective binding to the column and separation from other proteins. The bound EF-Tu and EF-G are then eluted from the thiol-Sepharose by a cysteine gradient. The EF-G is eluted first and EF-Tu later, well separated from each other [54].

Two approaches to prepare **EF-Tus of interest in a tagged form** to allow their selective extraction from the pool of all other bacterial proteins were adapted from tagging procedures described earlier.

It was shown that overproduced EcEF-Tu with a **C-terminal polyhistidine-tag** can be separated from wt EcEF-Tu on a Ni^{2+}-nitrilotriacetic acid-agarose (Ni-NTA-agarose) column [55]. The bound His_6-tagged EF-Tu is eluted from the column by washing with imidazole. A recent detailed protocol for a quick EF-TuHis purification on a small scale used in the laboratory of B. Kraal is given below. Purification on a larger scale is described in [56,57]. The C-terminal His_6-tag is usually not cleaved from EF-Tu for it was shown that it does not interfere with EF-Tu functions [58]. Preparation of bovine mitochondrial EF-Tu.GDP and EF-Ts by the polyhistidine tagging method is described in [59,60].

Finally, another successful method of EF-Tu tagging was with a protein-tag, the popular **glutathione-*S*-transferase (GST)** attached to the N-terminus of EF-Tu [61–63], using the pGEX cloning and expression system [64]. The GST-tagged EF-Tu is selectively fished from cell extracts by added Glutathione Sepharose 4B gel (the batch version of the procedure) or alternatively, similarly as in the case of His-tagging, bound to a small Glutathione Sepharose 4B column and thus separated from bacterial proteins. As a final step, the GST-fused EF-Tu bound to the resin is necessary to digest with an appropriate serine protease (blood clotting factor Xa) to cleave EF-Tu from the GST moiety. The factor Xa specifically recognizes the amino acid sequence Ile-Glu-Gly-Arg and cleaves downstream of it. Thus, either no or only a few (2–3) additional amino acid residues from the cloning site are left linked to the N-terminus of the protein of interest after cleavage depending on the type of the pGEX construct and cloning procedure. Another advantage is that the mutants expressed as a GST fusion are prevented from taking part in protein synthesis thereby circumventing problems with cell death and appearance of revertants (C.R. Knudsen, personal communication). A recent detailed protocol for the purification of particularly EF-Tu mutants by the column version of the procedure used in the laboratory of C.R. Knudsen, is given below. The GST technique was reported by Kim et al. [65] to be in their hands the only one suitable, from all other techniques tested in their laboratory, for the preparation of recombinant EF-Tu from *B. subtilis*.

Another GST fusion system with a thrombin site separating the GST moiety from EF-Tu has also been reported [66,67]. However, according to our experience, EF-Tu proteins seem to be more sensitive to unspecific thrombin cleavage than to treatment with factor Xa.

At present, almost all EF-Tu mutants and variants are expressed and purified by one of the two methods described above. In our laboratory, active *E. coli*, and *B. stearothermophilus* G-domains as well as six chimeric forms of EF-Tu composed of combination of individual domains of both factors were prepared by the batch version of the GST fusion technology via the pGEX system and characterized [11,12]. The details of the procedure have been described [68] and the most recent version of the procedure is given below. GST-tagged variants of *Bacillus subtilis* EF-Tu are currently characterized (H. Šanderová and J. Jonák, unpublished).

The large-scale purification procedures have been mainly developed for the purification of native EF-Tus whereas the small-scale affinity purification procedures for a rapid purification of both native EF-Tus and EF-Tu mutants and variants prepared by recombinant gene-engineering techniques. Their advantage is that they are simple, efficient, and require a relatively short time (1 to 2 days, see later).

4. Protocols and comments

4.1. The large scale EF-Tu purification protocols

In principle, EF-Tu is usually isolated from bacterial cell extracts as **EF-Tu·GDP** in essentially two fractionation steps and at 4 °C. The first step is an anion-exchange chromatography on DEAE-Sephadex A-50/DEAE-Sepharose FF/DEAE-Sepharose CL6B/DEAE-cellulose DE52/Q-Sepharose FF, and the second step is gel filtration on Sephadex G-100/Ultrogel AcA 44/Ultrogel AcA 54/Sephacryl S-200. The original isolation method of Lucas-Lenard and Lipmann from 1966 [69], primarily developed for native EcEF-Tu purification was further elaborated in Weissbach [13,14,19], and Kaziro laboratories [70] and simplified by Leberman et al. [71]. Separation of all three elongation factors from *Bacillus stearothermophilus* (Bst) by chromatography on DEAE-Sephadex A50 was first reported by the laboratory of Lengyel in 1968 [72]. For *E. coli* EF-Tu·GDP purification, Miller and Weissbach [13] introduced a third, crystallization, step. The crystallization of EF-Tu.GDP from the ammonium sulfate extracts further increased the purity of the preparation. Both the method of Miller and Weissbach [19] and that of Arai et al. [70] provided EF-Tu GDP protein pure enough to independently serve as a material for the successful determination of EcEF-Tu primary sequence in 1980 [73,74].

The above two-step scheme proved useful for isolating many bacterial EF-Tus. Due to special properties of some EF-Tus from different bacteria (p*I*, thermostatibility, etc.), some modifications to this scheme were also described.

EF-Tu can also be isolated from bacterial cell extracts in the form of **EF-Tu·Ts complex** and EF-Ts later displaced from EF-Tu by GDP (+Mg^{2+}) treatment. According to our own experience and that of others with the *E. coli system* and *B. stearothermophilus* system, to obtain the EF-Tu.EF-Ts complex, cell extraction and ion-exchange chromatography should be carried out in the absence of Mg^{2+} ions (see, e.g. [24,46,49]). In their presence, EF-Tu is always isolated in the EF-Tu·GDP form. This implies that there is a sufficiently high concentration of GDP and/or GTP in the crude cell extracts (the estimate is 0.3–1.0 mM GTP in rapidly growing cells, as reviewed in [75] and if Mg^{2+} ions (5–7 mM) are simultaneously present in isolation buffers, then the formation of the EF-Tu.EF-Ts complex is essentially prevented [13,70,71]. Nevertheless, to back-up this condition during preparation of EF-Tu in the GDP form, all buffers are usually supplemented with about 10 μM GDP [70].

A brief description of the procedure routinely used in our laboratory for the **purification of EF-Tu.GDP from *E. coli*** follows.

All steps are carried out at 0–4 °C. *Escherichia coli* cells (30 g) are suspended in 190 ml of buffer A (50 mM Tris/HCl, pH 7.6, 5 mM $MgCl_2$, 60 mM KCl, 5 mM 2-mercaptoethanol, 10 μM GDP, 10% glycerol, 1 mM PMSF) and sonicated 10 times for 20 s with 45-s brakes on ice to prepare cell extract. After centrifugation at about 30,000 × *g* for 40 min the resulting supernatant (S-30) is further centrifuged at about 150,000 × *g* for 2 h and the supernatant obtained (S-150) is diluted twice with buffer A without KCl and applied to a DEAE-Sepharose FF column (3 cm × 37 cm) equilibrated in buffer A with 50 mM KCl. The EF-Tu.GDP is eluted using a linear gradient of KCl (50–200 mM, 1500 + 1500 ml) in buffer A without PMSF at a flow rate 40 ml/h. The fractions (collected at 30 min intervals) containing EF-Tu.GDP are pooled and EF-Tu.GDP precipitated by solid ammonium sulfate to 55% saturation (with the simultaneous pH control). The sediment obtained after centrifugation is dissolved in 5 ml of buffer 20 mM Tris/HCl (pH 7.6) 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 10 μM GDP and 8.5% sucrose, and applied to a Sephadex G-100 column (4 × 100 cm) in buffer 20 mM Tris/HCl (pH 7.6) 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 100 mM KCl 10 μM GDP, 10% glycerol. The filtration proceeds at a flow rate of 20 ml/h. The void volume (about 500 ml) collected separately can be discarded and then the fractions (collected at 30 min intervals) containing EF-Tu.GDP are pooled and EF-Tu.GDP precipitated by solid ammonium sulfate to 55% saturation (with the simultaneous pH control). The sediment is successively extracted with 2.5, 1.5 and 1.5 ml of 42% saturated ammonium sulfate in buffer B composed of 20 mM Tris/HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol and 10 μM GDP. (The pH of the extraction solutions should be adjusted after addition of ammonium sulfate). The supernatants are discarded and the remaining sediment is extracted with 1.25 and 1.0 ml of 30% saturated ammonium sulfate in buffer B and finally with 0.6 ml of 20% saturated ammonium sulfate in buffer B. The EcEF-Tu.GDP protein readily crystallizes in long needles from 30%/20% saturated ammonium sulfate extracts if kept at 0–4 °C for several hours/days and the yield is increased by stepwise raising the ammonium sulfate saturation in the extracts to 40–45%. After about a month the crystals developed from all three extracts are collected by centrifugation, washed twice with 2 ml of the fresh 42% saturated ammonium sulfate solution in buffer B and stored in 35% saturated ammonium sulfate solution in the same buffer at 4 °C. Alternatively, the purified EF-Tu.GDP can be stored in a soluble form in buffer B with 50% glycerol at −20 °C. The yield of EF-Tu.GDP is about 50 mg.

Detection of EF-Tu in column fractions is carried out by the filter-binding assay (see above) using 10–20 μl aliquots. This is always accompanied with SDS-electrophoretic determination (with Coomassie Blue staining) of the composition of individual fractions and EF-Tu purity in particular. It is worth mentioning that the mobility on SDS gels of elongation factors Tu of essentially identical molecular mass but from different organisms may substantially differ. For example, the difference in the mobility between EcEF-Tu and BstEF-Tu repeatedly indicated that the latter protein should be by as much as 7 kDa larger than the former ([11], and references therein) despite the essentially same Mr of ∼43 kDa of both proteins (as determined from their amino acid sequences) [73,74,76]. This unexplained behavior appears to mainly stem from a mobility difference between their G-domains [11].

The separation of EF-Ts, EF-Tu.GDP and EF-G takes place already on the DEAE-Sepharose column, the proteins are eluted in the order indicated. EF-Ts is eluted well before EF-Tu.GDP, whereas EF-G slightly contaminates the last fractions of the EF-Tu.GDP peak. The full separation of EF-Tu.GDP from the

contamination by EF-G takes place on Sephadex G-100. This procedure (except for applying a 0.1–0.45 M KCl gradient for the elution from the DEAE-Sepharose FF column) was also used in our laboratory for the purification of EF-Tu.GDP from *Bacillus subtilis* (J. Jonák, unpublished).

In case the purity of EF-Tu fraction obtained after the gel filtration step is not satisfactory, an additional anion-exchange chromatography can be run or, with a small amount of the protein (0.5–1.0 mg), a purification on 1 ml FPLC (MonoQ) column can be recommended.

To prepare cell extracts several other methods besides the sonication (see also [10,70], etc.) were reported: passage through a French press [23,60,77], grinding of cells with twice bacteria mass of alumina (Al_2O_3) [42,44], lysis with lysozyme (EDTA, sodium deoxycholate, DNase I treatment) [32,54,59,62,71,78], etc.

In the simplification of the isolation procedure introduced by Leberman et al. [71] and widely applied also in other laboratories (e.g. [79]), the high speed centrifugation step to obtain the S-100/150 fraction and the crystallization step are avoided. The bacterial cell extract obtained after lysis of cell walls with 0.02% lysozyme and after treatment with 4% sodium deoxycholate and DNase I (1 mg/100 ml) followed by a low speed centrifugation is directly fractioned at pH 7.6 by ion-exchange chromatography on DEAE-Sepharose CL-6B. As reported, under these conditions, the separation of EF-Tu·GDP from EF-G factor from extracts obtained from three different bacterial organisms, *E. coli, Bacillus stearothermophilus* and PS3 did not occur well. In fact, both factors from the two thermophilic bacteria were eluted together. Two long AcA 44 gel filtration columns connected in series were then required to separate EF-Tu.GDP from EF-G only on the basis of their different molecular weights. Therefore, in some laboratories ammonium sulfate back-extraction and crystallization (if possible) are included as a final step. According to our experience, ***B. stearothermophilus*** **factors EF-Tu·GDP** and EF-G can be relatively well separated from each other already in the first chromatographic step by decreasing the pH of the chromatography to about 6.5 as originally shown using a DEAE-Sephadex A-50 column [80]. With a gradient of 0.15–0.55 M KCl in a buffer containing 0.02 M sodium cacodylate (pH 6.5), 0.01 M $MgCl_2$, 5 mM2-mercaptoethanol, 1 mMNaN_3, 10 μM PMSF with or without 10 μM GDP, the factors EF-Ts, EF-G and EF-Tu emerge from the column at about 0.365, 0.4 and 0.435 M KCl, respectively. Note, that by lowering the pH, EF-G is eluted from the ion-exchange column before EF-Tu·GDP. An analogous separation of EF-Tu.GDP from EF-G and EF-Ts can be obtained by a chromatography on a DEAE-Sepharose FF column at pH 6.8 and using a gradient of 0.1–0.45 M KCl in the above buffer supplemented with 10% glycerol or without glycerol (Fig. 2). The EF-Tu·GDP containing fractions are in both cases further separated from traces of EF-G by gel filtration chromatography on a Sephadex G-100 column (4 × 110 cm) (Fig. 3). The pure EF-Tu.GDP is precipitated by solid ammonium sulfate to 55% saturation, the sediment dissolved in buffer B with 10% glycerol and dialyzed against the same buffer. Undissolved aggregates are removed by centrifugation and the supernatant stored at −20 °C. The yield was usually about 70 mg of EF-Tu.GDP from 30 g of wet cells. Separation of active ***B. stearothermophilus*** **EF-Tu.Ts complex** from EF-G on a DEAE-Sephadex A50 column at pH 6.5 (in a Tris–maleic acid buffer without magnesium ions) has been also described [24].

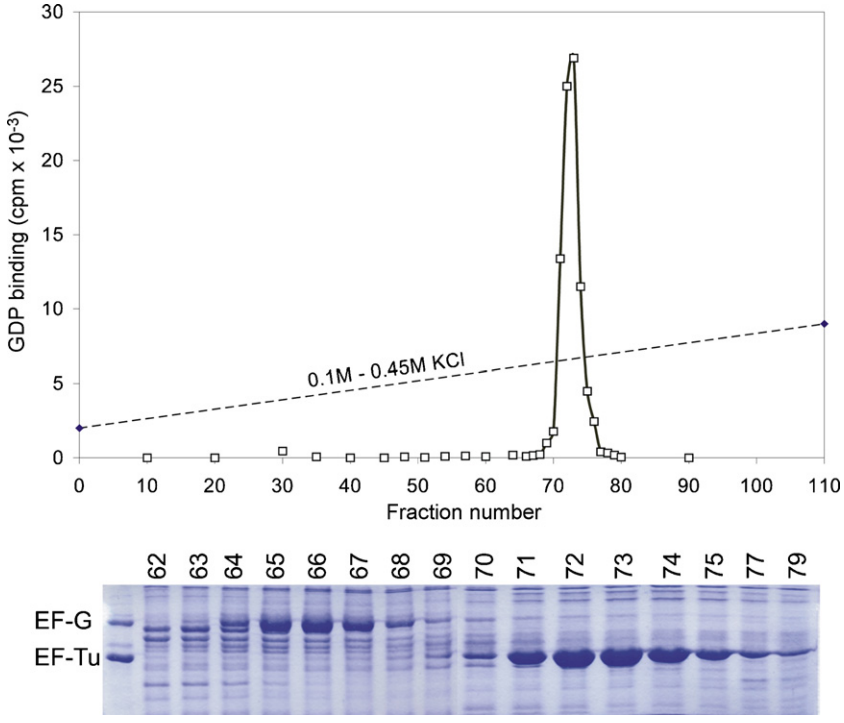

Fig. 2. Separation of *B. stearothermophilus* EF-Tu.GDP from EF-G by DEAE-Sepharose FF chromatography step (see text for details). Binding of [^{3}H]GDP (□) to detect EF-Tu and SDS-polyacrylamide gel electrophoretic analysis of some fractions are shown. The position of EF-Tu and EF-G is indicated.

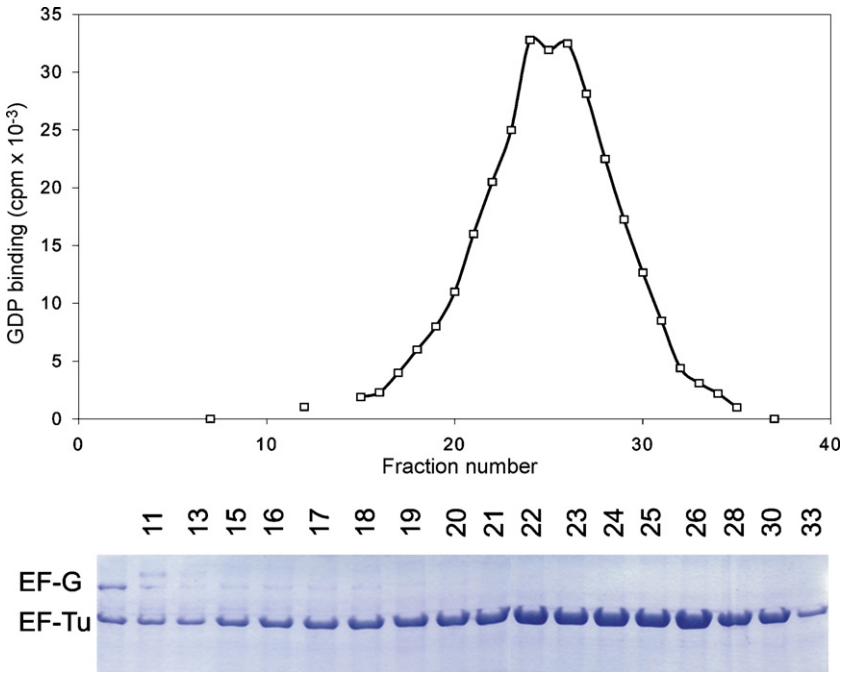

Fig. 3. Purification of *B. stearothermophilus* EF-Tu.GDP by gel filtration on a Sephadex G-100 column (see text for details). Binding of [^{3}H]GDP (□) to detect EF-Tu and SDS-polyacrylamide gel electrophoretic analysis of some fractions are shown. The position of EF-Tu and EF-G is indicated.

EF-Tu.GDP from *Streptomyces aureofaciens* has the ability to spontaneously aggregate when concentrated from partially purified solution at 0–4 °C. This was shown to simplify its purification. No gel filtration chromatography step is required [81]. The recent protocol for *S. aureofaciens* EF-Tu.GDP purification routinely used in the laboratory of J. Weiser is following: Fractions containing EF-Tu.GDP collected during a regular chromatography of an extract from *S. aureofaciens* cells (100 g) on a DEAE-Sephadex column (3 × 28 cm) with a linear 0.1–0.4 M KCl (500 + 500 ml) gradient in a standard buffer (50 mM Tris/HCl pH 7.4, 10 mM $MgCl_2$ 10 mM 2-mercaptoethanol and 10 μM GDP) are combined and EF-Tu.GDP precipitated by solid ammonium sulfate to the final concentration of 56.8 g/100 ml (about 36% saturation). The precipitate is dissolved in about 5 ml of a standard buffer with 100 mM KCl, denatured proteins removed by centrifugation and the supernatant is dialysed extensively against the above buffer to remove ammonium sulfate. The content is then concentrated by repeatedly immersing the dialysis tubing into dry powder of Sephadex G-200 to the final volume of about 2–3 ml. At this step EF-Tu aggregates as a white glittering suspension, it is pelleted, washed in a standard buffer with 100 mM KCl to remove non-aggregated proteins and finally stored in the same buffer in a freezer. The protein is electrophoretically pure and it is active in GDP binding and polyphenylalanine synthesis.

To prepare large amounts of ***T. aquaticus* EF-Tu** for crystallization and X-ray studies, the purification was started by chromatography on Q-Sepharose FF, followed by gel filtration on AcA 54 Ultrogel and by a HPLC hydrophobic chromatography on TSK-Phenyl 5W bed using a back gradient of ammonium sulfate [77]. The hydrophobic chromatography on TSK-Gel Phenyl 5PW with a reversed gradient of ammonium sulfate was shown to efficiently **separate the binary complexes of *T. thermophilus* EF-Tu.GDP, and EF-Tu.GDPNP as well as the ternary complex EF-Tu.GDPNP.Leu-tRNA from each other** [82].

Purification of **EF-Tu from *T. thermophilus*** on Q-Sepharose FF was also described [32].

Purification of **SELB-selenocysteyl-tRNA^{UCA} binding elongation factor** replacing the common EF-Tu in a special step of UGA codon translation into selenocystein is described in [83].

4.2. Large scale purification of G-domains of EF-Tus

G-domains (with no tagging) of bacterial EF-Tus overproduced in *E. coli* cells from various expression plasmids (e.g. pFLAG-CTC) in native, point-mutated or chimeric form can be efficiently purified by the same chromatographic procedure as described above for the purification of EF-Tu.GDP from *E. coli* (EcEF-Tu.GDP). If the p*I* of the G-domain differs from that of EcEF-Tu their mutual separation already occurs during the first chromatography on an anion-exchanger. Final purification is achieved in the gel filtration step on Sephadex G-100. By this way (except for applying 80–250 mM KCl gradient in the chromatography on DEAE-Sepharose FF) we were able to purify overproduced G-domains of *B. subtilis* and *B. stearothermophilus* EF-Tus. Due to the fact that p*I* of the G-domains is lower (p*I* ∼ 4.74 and 4.82, respectively) than that of EcEF-Tu (p*I* ∼ 5.30) and EF-G from *E. coli* (p*I* ∼ 5.24) the G-domains are eluted from the anion-exchanger later, at a higher concentration of KCl than the factors (Fig. 4, J. Jonák and H. Šanderová, unpublished). In this case the yield was

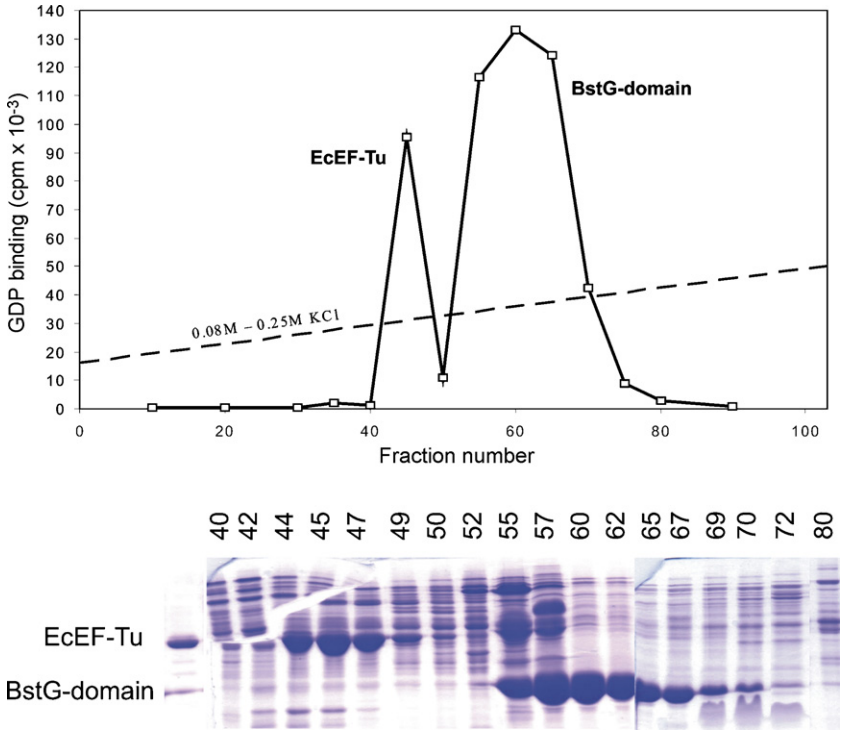

Fig. 4. Separation of *B. stearothermophilus* G-domain (BstG-domain) overproduced in *E. coli* from *E. coli* EF-Tu (EcEF-Tu) and partially from *E. coli* EF-G by chromatography on a DEAE-Sepharose FF column (see text for details). Binding of [^{3}H]GDP (□) to detect *E. coli* EF-Tu and *B. stearothermophilus* G-domain, and SDS-polyacrylamide gel electrophoretic analysis of some fractions are shown. The position of *E. coli* EF-Tu and *B. stearothermophilus* G-domain is indicated.

about 230 mg of purified G-domain of *B. stearothermophilus* EF-Tu from 30g of wet *E. coli* cells. Analogous purification procedures of overproduced G-domains using Q-Sepharose or DEAE-cellulose DE52 columns were also described [10,42,84–86].

4.2.1. Technical comments

DEAE-Sepharose FF and DEAE-Sephadex A-50 have had in our hands very similar resolution capacities. The only difference was that the elution of the factors from the former bed takes place at lower KCl concentrations than from the latter one. However, the use of DEAE-Sepharose/Q-Sepharose is preferable to that of DEAE-Sephadex mainly because the regeneration of the DEAE-Sepharose is remarkably easy and quick in comparison to that of DEAE-Sephadex. First of all, it can be done directly in the column, simply by thorough washing with 3 M KCl (according to the instructions of the manufacturer). It is then followed by equilibration with the starting buffer and the column is prepared for the next run. The regeneration procedure can be repeated many times, directly in the cold room, without any apparent loss of activity of the bed (J. Jonák and A. Parmeggiani, unpublished results). We found very useful to also regenerate Sephadex G-100 after each run. The reason is to remove traces of contaminants with proteolytic activity that stay sticking to the gel even after thorough washing with a high ionic strength buffer. This activity can split the susceptible and conserved bond Arg58-Gly59 (according to *E. coli* numbering) in the G-domain of EF-Tu. *B. stearothermophilus* EF-Tu is particularly sensitive to this action (J. Jonák, unpublished results). The contaminants with the proteolytic activity can be efficiently removed by washing of Sephadex G-100 with an excess of 0.5 M NaOH in a glass cylinder at room temperature for about 1 h. The decanted slurry is then washed by destilled water to neutral pH and finally equilibrated in the column buffer. This time the washing should be most easily done with an unpacked Sephadex bed so that a new packing of the column with Sephadex is required after every regeneration cycle.

4.3. The small scale EF-Tu purification protocols

4.3.1. Isolation of C-terminally His-tagged EF-Tu

Growth: inoculate fresh cells in rich medium (LC for *E. coli* and TSBS for *Streptomyces coelicolor*).

For inducible expression, grow until an OD of ∼0.4 and induce during 3 h under vigorous shaking. For constitutive expression (*S. coelicolor*), grow for ∼48 h.

Harvest cells by centrifugation at 4000 rpm for 15 min at 4 °C. Use immediately or keep at −20 °C until use.

Cell disruption: resuspend cell pellet in buffer containing 10 μM GDP. For *E. coli* buffer I (50 mM Tris–HCl (pH 7.5), 60 mM NH_4Cl and 7 mM $MgCl_2$) was used. For isolation of either EF-Tu1 or EF-Tu3 from *S. coelicolor*, cell pellets are resuspended in an equal volume of buffer A (1×PBS, 1% (w/v) sarkosyl, 1% (v/v) triton X100) and buffer B (20 mM Tris–HCl (pH 7.5), 150 mM NaCl).

Cells are lysed by means of sonication (5 s on and 5 s off) during 10 min at 4 °C.

S30 fraction is isolated by a single ultracentrifugation step (30 min at 30,000 rpm at 4 °C).

Isolation of EF-Tu: Cell-free extract is incubated with 0.25 volume of Ni^{2+}-NTA slurry (Qiagen) by head-over-tail rotation at room temperature during ~20 min and then applied to a disposable empty column (Qiagen). All further washings are done at room temperature by applying 2 ml of the different buffers and let it flow by gravity while avoiding that the matrix runs dry. The buffers used all are based on buffer I and contained 0 mM, 5 mM, 10 mM, 20 mM, 50 mM, 300 mM, or 500 mM imidazole [a 2 M, buffered (pH 7.5) imidazole solution was used for making these solutions]. Elution profile and purity are checked by SDS-PAGE and Coomassie staining. Typically, EF-Tu elutes at 300 mM and 500 mM imidazole. Purity is estimated to be higher then 95%. All together this isolation procedure takes 20 min of immobilization to the Ni^{2+} matrix and 10 min for washing and elution.

4.3.2. Expression and purification of EF-Tu by the GST-method

4.3.2.1. Column version (according to C.R. Knudsen). In principle, any *E. coli* strain can be used for overexpression. Usually the strain JM109 is used, which is *recA* deficient, thereby eliminating the risk of homologous recombination resulting in the loss of the point-mutation. All cultures are grown in 2×TY containing ampicillin (100 mg/l). An overnight culture is made from a colony obtained from freshly transformed cells (less than 1 week old). Expression cultures are inoculated with 1% overnight culture and grown at 28 °C until OD 0.6–0.8 is reached. IPTG is added to 0.1 mM and the culture is incubated for another 3 h. The cells are harvested by centrifugation and frozen at −20 °C. The overexpressed protein can be released from the cells by treatment with lysozyme, passage through a French press or by sonication. The resulting cell lysate is clarified by centrifugation (10,000 × *g*, 10 min, 4 °C) before application to the column. Prior to sample application, the glutathion agarose column is equilibrated with buffer I (50 mM Tris–Cl, pH 7.6 (4 °C); 10 mM $MgCl_2$; 15 μM GDP; 1% Triton X-100). The cell lysate is diluted with an equal volume of buffer I. The sample is loaded on the column at 0.5 ml/min. In our hands, the glutathione agarose column material available from Sigma has got the highest-binding capacity for GST-EF-Tu.

The column is washed with buffer II (50 mM Tris–Cl, pH 7.6 (4 °C); 10 mM $MgCl_2$; 15 μM GDP). The fusion protein is eluted with buffer II containing 5 mM-reduced glutatione (pH of the buffer should be adjusted after addition of glutathione, which is acidic). The relevant fractions identified by SDS-PAGE are pooled and dialysed against the cleavage buffer (50 mM Tris–Cl, pH 7.6 (4 °C); 10 mM $MgCl_2$; 15 μM GDP; 100 mM NaCl). The protein concentration of the dialysed fractions is determined. It should be at least 1 mg/ml to ensure efficient cleavage. Otherwise, the protein solution is concentrated to reach the appropriate concentration. The cleavage reaction is started by adding factor Xa (1/300 of the weight of GST-EF-Tu) and allowed to proceed overnight at 4 °C. The proteolytic reaction is stopped by addition of 0.5 mM PMSF, which irreversibly inhibits serine proteases. The cleavage reaction is applied on the washed column and EF-Tu is collected in the flow-through.

4.3.2.1.1. Comments. It is worthwhile doing analytical cleavage tests before proceeding to the preparative scale. The ratio between factor Xa and fusion protein (in weight) should be varied (try e.g. 1:200, 1:400 and 1:600) and samples withdrawn at different time intervals (between 4 h and overnight). 10% glycerol can be added to the fusion protein to allow freezing, while doing the cleavage optimisation. The glycerol slows down the cleavage (and often makes it more specific). Factor Xa from Qiagen can be recommended, after comparing the activity of the proteases from various sources.

The system has been successfully applied to a large number of point-mutants of EF-Tu. The system is quite robust, but a couple of problems have been noticed. In some cases, the yield of soluble protein upon expression is low due to the formation of inclusion bodies. This problem has been circumvented to a large extent by growing the expression cultures in a medium containing sorbitol and betaine [87]. Others have prevented similar problems by co-expressing EF-Ts (see above). Some mutants have caused problems during proteolytic cleavage, when other sites apart from the canonical factor Xa site are cleaved. The susceptible sites are expected to be arginines 44 and 58 flanking the effector region. Seemingly, the problematic mutations may cause structural changes, which manifest themselves in a more accessible effector loop. This problem can be reduced by performing the abovementioned optimisation of the cleavage reaction. In this case, it is particularly useful to include 10% glycerol in the reaction. Others have reported unspecific cleavage by factor Xa after Gly-Arg dipeptides. In the case of *E. coli* EF-Tu, the susceptible arginines are preceded by alanines.

4.3.2.2. Batch version. Preparation of bacterial crude extract: three hundred ml (see comments below) of the rich RMK medium [68] were supplied with ampicilin (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 $A_{600} = 0.8$, then cooled down to 20 °C and 100 mM IPTG was added to the final concentration 0.1 mM and the incubation continued for 3 h at 20 °C. 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 (1×PBS, 10 mM $MgCl_2$, 7 mM 2-mercaptoethanol, 10% glycerol and 15 μM GDP) and disrupted by sonication (6 × 10 s with 2 min interval at 4 °C). Twenty percent Triton X-100 was added to the final concentration of 1% and the suspension was incubated on ice with permanent shaking for 30 min, then twice centrifuged at 12,000 × *g* to remove cell debris. Supernatant was retained for the next step.

Binding of GST-fused proteins to Glutathione Sepharose 4B beads: six hundred μl of 50% Glutathione Sepharose 4B (GS4B; bead suspension, prepared according to the instructions of the manufacturer) were mixed with 15 ml of the supernatant and incubated on ice with permanent shaking for 1 h. The suspension was centrifuged and the sedimented GS4B beads carrying the bound fusion protein were washed four times with 15 ml buffer A and once with 15 ml buffer B (50 mM Tris–Cl, pH 7.6, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 7 mM 2-ME, 10% glycerol and 15 μM GDP).

Factor Xa cleavage and isolation of GST-free protein: two different strategies were applied. If recombinant GST-proteins involved G-domain of *E. coli* EF-Tu, then 450 μl of buffer B and 4 U of factor Xa (1 U/μl) from Amersham were mixed with the Glutathione Sepharose 4B-bound fusion protein suspension and the suspension was incubated at 4 °C for 16 h. If recombinant GST-proteins involved G-domains of *B. stearothermophilus* or *B. subtilis* EF-Tu, then 450 μl of buffer B and 60 U of factor Xa (1 U/μl) from Amersham were mixed with the Glutathione Sepharose 4B-bound fusion protein suspension and the suspension was incubated at 8 °C for 1 h (see comments below for explanation). The cleavage reaction was stopped by addition of PMSF to 1 mM concentration; in the reactions involving EcG-domain the Xa Removal Resin (Qiagen) was alternatively used. 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 and stored at −20 °C.

4.3.3. Comments

The overexpressed form of GST-G-domain of *E. coli* (in contrast to GST-EcEF-Tu, GST-EF-Tu from *B. stearothermophilus* or chimeric GST-forms of EF-Tu including those in which the EcG-domain was present) was mainly obtained in an insoluble form, in inclusion bodies [68]. Therefore, the preparation started from 900 ml (instead of 300 ml) of induced cell culture and the overexpressed GST-G-domain was isolated from a 100,000 × *g* supernatant of sonicated cell crude extract.

Similarly, as described in the comments to the column version of the GST procedure described above, the integrity of the susceptible, Arg-Gly bond should be carefully checked during factor Xa treatment. Moreover, its susceptibility also depends on the origin of the factor. We found out that this bond is in G-domains of *B. stearothermophilus* and *B. subtilis* EF-Tu (they both have Glu57-Arg58-Gly59 at this position) much more sensitive to factor Xa unspecific cleavage than it is in EcG-domain (with Ala57-Arg58-Gly59 at this position). This explains two different strategies we apply when preparing GST-fused EF-Tus and G-domains from these organisms. In our hands, the unspecific cleavage at the susceptible Arg-Gly bond occurred less frequently with factor Xa from Amersham than from Qiagen.

Naturally, if required, the "small" scale purification protocols can be reasonably scaled up.

5. Preparation of nucleotide-free EF-Tu and EF-Tu.GDPNP

Nucleotide-free EF-Tu is required for some kinetic measurements and as a starting molecule for preparation of binary complexes of EF-Tu with various guanosine nucleotides. To prepare the nucleotide-free EF-Tu several methods have been described. In the classical one, developed primarily for EcEF-Tu, the bound GDP in EF-Tu.GDP was first converted to GTP with a GTP regeneration system. GTP binds to EF-Tu with about thousandfold lower affinity than GDP. Then magnesium ions were removed by EDTA to completely brake down EF-Tu-GTP interactions (see above). The dissociated GTP was removed by gel filtration chromatography on Sephadex G-25 and EF-Tu was eluted in the void volume essentially free of any bound nucleotide [88,89]. Due to inherent instability of free EF-Tu the percentage of active molecules in the preparation did not usually exceed about 30%.

This strategy was greatly optimized and the isolation time much shortened by introduction of Chromaspin TE 10 spin columns (Clontech) + centrifugation instead of Sephadex G25 column, as described in [62,90].

In another method, GDP in EF-Tu.GDP is completely digested by bovine/intestinal alkaline phosphatase and EF-Tu readily forms a complex with added, e.g. GDPNP ([91,92]; originally developed for p21 protein [93]). The high efficiency of this converting method was proved by obtaining a crystallizable ternary complex EF-Tu.GDPNP.aminoacyl-tRNA [23] from EF-Tu.GDPNP prepared by the phosphatase method. To crystallize natural EF-Tu.GTP complex for structural studies is not possible due to intrinsic GTPase activity of EF-Tu.

6. Conclusions

Classical column chromatography methods offer a reasonable way to prepare large (mg) amounts of natural EF-Tus or their engineered G-domains from various bacteria in a pure state and a relatively short time (about 10 days). These large scale procedures are complemented by a set of highly selective small scale procedures, which can be applied using commercially available kits, allowing now relatively easy and rapid (in a few days) preparation of essentially any pure EF-Tu and its variants. Nevertheless, it is necessary to bear in mind that despite a great degree of homology in their amino acid sequences, individual elongation factors EF-Tu and especially their variants can differ in their p*I*, thermostability, solubility, folding capacity, affinity for guanine nucleotides, susceptibility of their peptide bonds to various agents, etc. This will always require appropriate adaptation of the procedures for their isolation.

The results obtained on EF-Tu proteins are meaningful for the whole family of GTP-binding proteins. The next step will be now to correlate not yet understood functional findings with structural data. Study of the reversible and energy-dependent conformational transitions of the functional protein such as EF-Tu are of prime importance for the understanding of energy signal transduction pathways in biological systems, cell physiology and drug action.

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