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

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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.

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

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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_2HPO_4, 18 m*M* KH_2PO_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% $MgSO_4 \cdot 5H_2O$ (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).

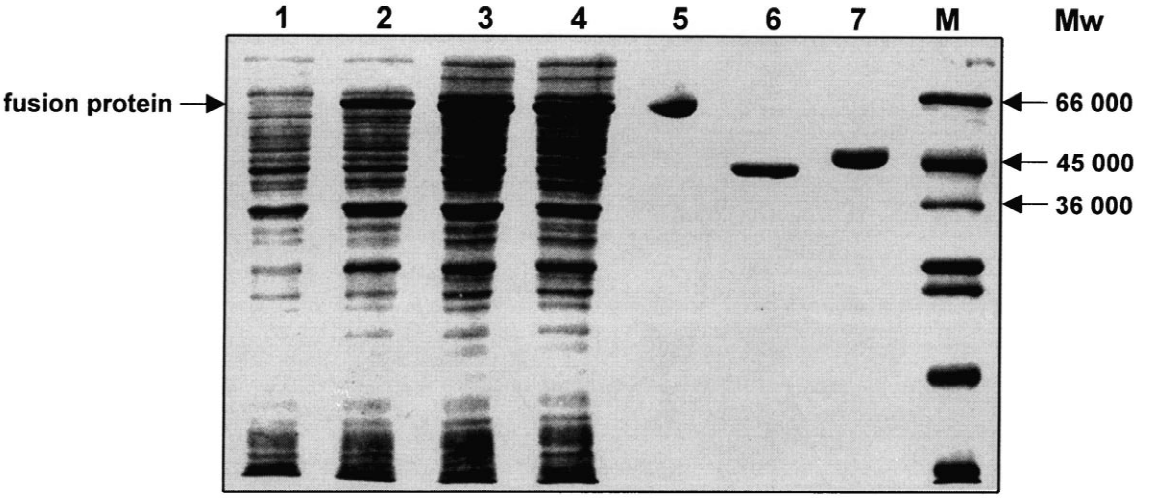

Fig. 1. Expression and purification of chimaeric EF-Tu (CH1). 12% SDS–PAGE stained with Coomassie Brilliant Blue. Non-induced cell culture (lane 1), cell culture after IPTG induction of expression of the GST–CH1 fused protein (lane 2), bacterial lysate (lane 3), supernatant for binding of the fusion 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].

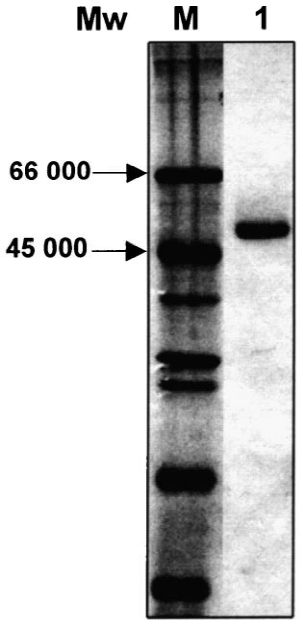

Fig. 2. 12% SDS–PAGE of GST–*Bst* G-domain fusion protein (stained with Coomassie Brilliant Blue). M-protein markers.

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 μ*M* GDP and 10 m*M* 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

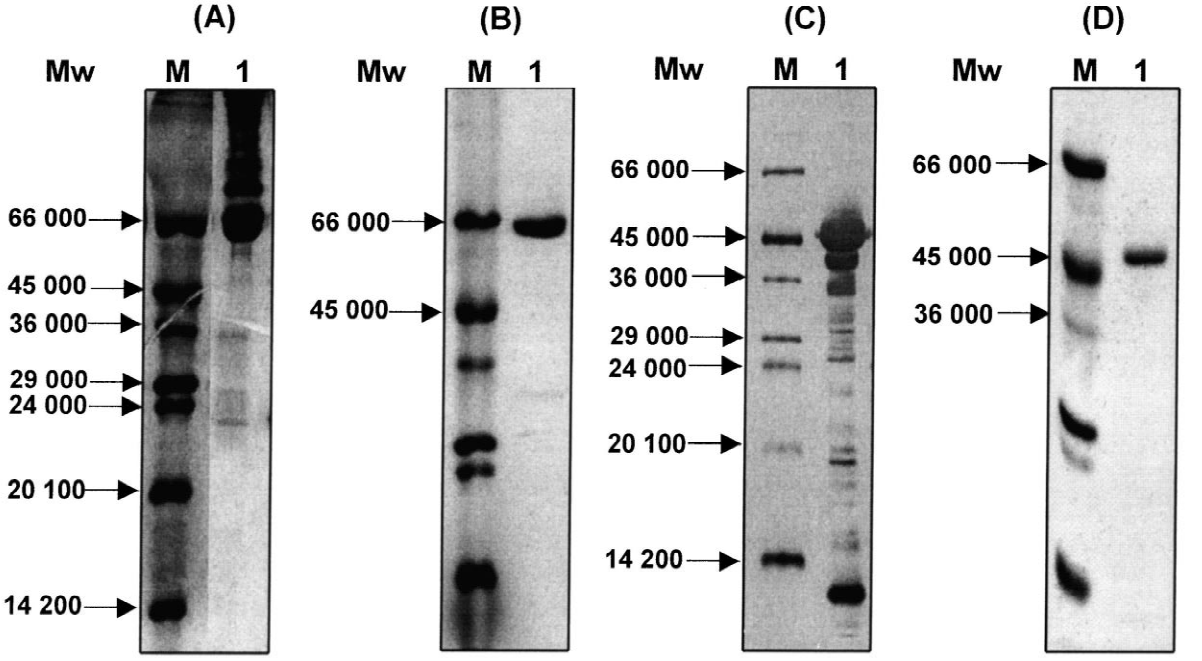

Fig. 3. Purification of EF-Tu proteins by the standard and by the modified GST-procedure: a comparison. GST–CH4 fusion protein (lane 1) purified by the standard (A) or by the modified procedure (B). GST-free recombinant EF-Tu from *B. stearothermophilus* (lane 1) purified by the standard (C) or by the 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

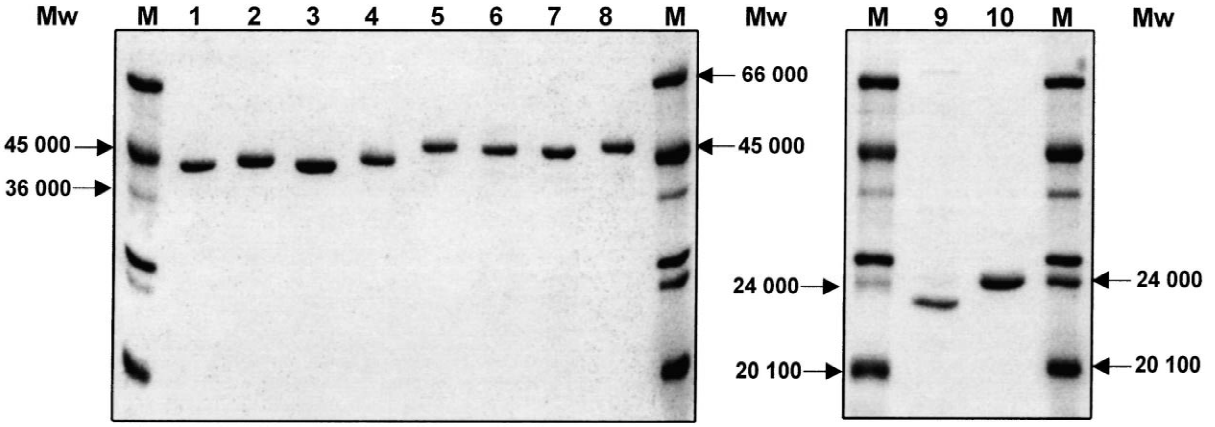

Fig. 4. 12% SDS–PAGE of isolated proteins (stained with Coomassie Brilliant Blue). *Ec* rEF-Tu (lane 1), CH1 (lane 2), CH2 (lane 3), CH3 (lane 4), *Bst* rEF-Tu (lane 5), CH4 (lane 6), CH5 (lane 7), CH6 (lane 8), *Ec* G-domain (lane 9) and *Bst* G-domain (lane 10). M-protein markers.

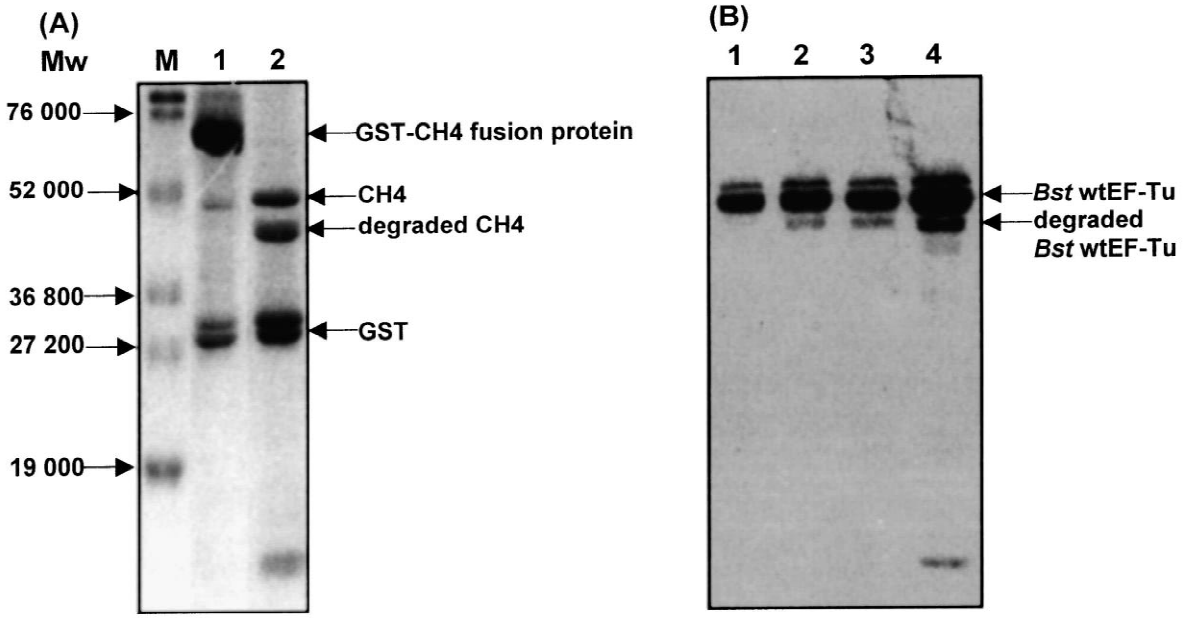

Fig. 5. Degradation of EF-Tu by prolonged factor Xa treatment. (A) Non-treated fusion protein (GST–CH4) (lane 1); fusion protein GST–CH4 treated with factor Xa at 8°C overnight (lane 2). (B) EF-Tu (*B. stearothermophilus*) treated with factor Xa at 8°C for 30 min (lane 1), 60 min (lane 2), 90 min (lane 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 m*M* 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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References

[1] I.M. Krab, A. Parmeggiani, Biochim. Biophys. Acta 1443 (1998) 1.
[2] L. Krásný, J.R. Mesters, L.N. Tieleman, B. Kraal, V. Fučík, R. Hilgenfeld, J. Jonák, J. Mol. Biol. 283 (1998) 371.
[3] M.D. Jones, T.E. Petersen, K.M. Nielsen, S. Magnusson, L. Sottrup-Jensen, K. Gausing, B.F.C. Clark, Eur. J. Biochem. 108 (1980) 507.
[4] M. Kjeldgaard, J. Nyborg, B.F.C. Clark, FASEB J. 10 (1996) 1347.
[5] A. Parmeggiani, G.W.M. Swart, K.K. Mortesen, M. Jensen, B.F.C. Clark, L. Dente, R. Cortese, Proc. Natl. Acad. Sci. USA 84 (1987) 3141.
[6] M. Jensen, R.H. Cool, K.K. Mortensen, B.F.C. Clark, A. Parmeggiani, Eur. J. Biochem. 182 (1989) 247.
[7] D.B. Smith, K.S. Johnson, Gene 67 (1988) 31.
[8] C.R. Knudsen, B.F.C. Clark, B. Degn, O. Wiborg, Biochem. Int. 28 (1992) 353.
[9] R. Cetin, P.H. Anborgh, R.H. Cool, A. Parmeggiani, Biochemistry 37 (1998) 486.
[10] S.I. Kim, H.Y. Kim, S.H. Kwon, S.Y. Lee, Mol. Cells 10 (2000) 102.
[11] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[12] U.K. Laemmli, Nature 227 (1970) 680.
[13] P.H. Anborgh, A. Parmeggiani, J. Jonák, Eur. J. Biochem. 208 (1992) 251.
[14] H. Tomincová, M. Brabcová, L. Krásný, J. Jonák, Chem. Listy 94 (2000) 548.