An intact conformation at the tip of elongation factor G domain IV is functionally important

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Abstract Three variants of *Thermus thermophilus* EF-G with mutations in the loop at the distal end of its domain IV were obtained. The replacement of His-573 by Ala and double mutation H573A/D576A did not influence the functional activity of EF-G. On the other hand, the insertion of six amino acids into the loop between residues Asp-576 and Ser-577 reduced the translocational activity of EF-G markedly, while its GTPase activity was not affected. It is concluded that the native conformation of the loop is important for the factor-promoted translocation in the ribosome. The functional importance of the entire EF-G domain IV is discussed.

Key words: Ribosome; Elongation factor G function; Mutagenesis; Translocation

1. Introduction

The elongation phase of polypeptide chain synthesis in bacteria requires two elongation factors (EF), EF-Tu and EF-G. Their eukaryotic counterparts are EF-1 and EF-2. EF-Tu participates in the codon-dependent aminoacyl tRNA binding to the ribosomal A site and EF-G stimulates translocation, i.e. the peptidyl tRNA movement from the A site to the P site. During translocation the displacement of mRNA by one codon is coupled to tRNA transfer [1].

The tertiary structures of EF-Tu and EF-G with different ligands have been determined (for review see [2,3]). The structural comparison of the EF-Tu*aminoacyl-tRNA*GTP ternary complex and EF-G revealed that domains III, IV and V of EF-G mimic the tRNA moiety of the ternary complex. Domain IV of EF-G resembles the anticodon helix of tRNA and is thought to be important for translocation [4]. The functional importance of amino acid residues located at the distal end (tip) of domain IV has been discussed [2,5,6].

In the present communication we describe some properties of three variants of *Thermus thermophilus* EF-G with mutations at the tip of domain IV. The results indicate that the intact conformation of the loop at the tip of domain IV is essential for EF-G function.

2. Materials and methods

Restriction endonucleases and T4 DNA ligase were from Promega. DNA polymerase was purchased from New England Biolabs and used according to the manufacturer's directions. Resins for protein isolation, Q-Sepharose FF, Ultrogel Ac34 and HA-Ultrogel, were from Pharmacia.

For gene expression plasmid pET11c and *Escherichia coli* strains BL21(DE3) or B834(DE3) (Novagen) were used [7]. All recombinant DNA procedures and cell growth were carried out according to the published manual [8]. The plasmid pLS3 with the *T. thermophilus* EF-G gene was a gift from Dr. N.I. Matvienko (Institute of Protein Research).

2.1. Primers

Oligonucleotides were synthesized by Gene Assembler Plus (Pharmacia) according to the manufacturer's manual. All primers are given in the 5′-3′ direction. Pr1, CGG TGGTGCATATGGCGGTCAAGGTAG, contains the restriction site *Nde*I and is complementary to the 5′-end of the EF-G gene. Pr2, GCTCCTAC**GC**CGAGGT**C**GACTCCTCC, primer for H573A mutation with the *Sal*I site. Pr3, GCGAATTCTATTGACCCTTGATGAGC, primer with the *Eco*RI site, is complementary to the 3′-end of the gene. Pr4, GGCTC**A**TA**TGC**CGAGGTGG**C**CTCCTCCGA, primer for double mutation H573A/D576A with the *Nde*I site. Pr5, TCGACGGTACCGGCTCCG and Pr6, TCGACGGAGCCGGTACCG, are oligonucleotides with the *Kpn*I site and were used for insertion mutation. Primers Pr5 and Pr6 are complementary to each other and contain the overhangs for endonuclease *Sal*I at their 5′-ends. The restriction sites are underlined, non-complementary nucleotides are given in bold letters.

2.2. Mutagenesis

The H573A mutation was created by polymerase chain reaction (PCR). Plasmid pLS3 carrying the *fus* gene from *T. thermophilus* was used as a template. The DNA fragment obtained with Pr2 and Pr3 (about 400 bp) in the first round of PCR was used as the 3′-end megaprimer in the second PCR round together with Pr1. The final PCR product was treated with endonucleases *Nde*I and *Eco*RI and cloned into plasmid pET11c at the corresponding sites. As a result, plasmid pETH containing a mutated *fus* gene was obtained. The mutation was verified by the appearance of a new unique *Sal*I site. PCR reactions were carried out as published in [9].

Double mutation H573A/D576A was obtained in a similar way using primers Pr4 and Pr3 in the first PCR reaction. After the second PCR round the PCR product was digested with enzymes *Hin*dIII and *Eco*RI and used for replacement of the corresponding DNA fragment in plasmid pETH. By this means plasmid pETHD was constructed. Mutation was confirmed by the appearance of a new *Nde*I site.

The insertion mutation was made with oligonucleotides Pr5 and Pr6. They were annealed together and the duplex was cloned into plasmid pETH digested with the *Sal*I enzyme. Thus plasmid pETL with the inserted DNA fragment (18 bp) was obtained. The construction was verified by the appearance of a unique *Kpn*I site. Correct orientation of the insert was checked by PCR using primers Pr1, Pr6 or Pr3 and Pr5.

2.3. Gene expression and protein isolation

Cells were grown in LB broth. Induction with isopropylthiogalactoside was done at $OD_{590} = 0.8–1.0$ o.u. of cell suspension. The proteins were isolated by successive use of ion-exchange chromatography on Q-Sepharose FF (Pharmacia), heat denaturation of *E. coli* proteins at 72°C and gel filtration on Ultrogel AcA34 [10]. The purity of the isolated proteins was checked by SDS-PAGE [11].

2.4. Functional tests

Poly(U)-directed polyphenylalanine synthesis was carried out in a

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cell-free translation system with purified elongation factors Tu and G according to the published procedure [12].

Ribosome-dependent hydrolysis of GTP was carried out in a 100 μl mixture containing 0.4 mM GTP, 3 mM phosphoenolpyruvate, 20 pmol of ribosomes, 0.5 pmol of EF-G and 0.5 μg/ml pyruvate kinase. The EF-G activity was determined by measuring the released inorganic phosphate spectrophotometrically as in [13].

Puromycin reaction was carried out with 45 pmol of the ribosomes (38 pmol of [^{14}C]PhetRNA bound), 40 pmol of the EF-G and 1 mM puromycin as in [14].

3. Results and discussion

Three mutated variants of *T. thermophilus* EF-G were obtained as a result of site-directed mutagenesis. All mutations were done in the loop connecting β-strand 7 and helix B (amino acid residues 573–579) [5,6] (see Fig. 1). The structural motif YHEVDS in the loop is highly conserved among bacterial EF-G [15] and might be functionally important [5,6]. For this reason His-573 in the first construct was changed to Ala (H573A) and the second mutated protein contains the double mutation H573A/D576A. The third mutation was constructed by insertion of a short DNA fragment into the *fus* gene with the H573A mutation. Thus, it comprises mutation H573A as well as inserted peptide GTGSVD between residues Asp-576 and Ser-577.

All mutated EF-G genes were expressed in *E. coli* cells and the proteins were produced in soluble form. The mutated factors retained high thermostability, and this facilitated their purification. The yield of purified proteins was about 7 mg/l of cell culture. According to electrophoresis the purity of the isolated proteins was better than 95% (Fig. 2).

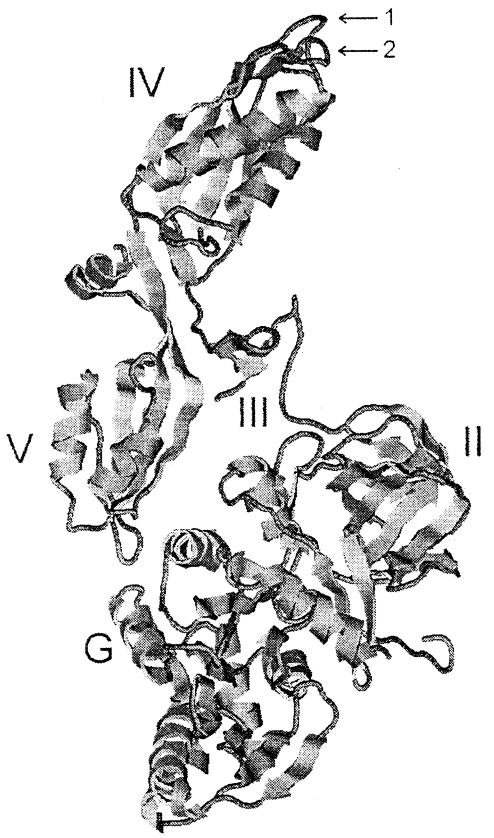

Fig. 1. Ribbon model of the tertiary structure of intact *T. thermophilus* EF-G. Arrows indicate the loops at the tip of domain IV: 1: residues 573–579; 2, residues 501–504. The model was drawn with the help of the RASMOL program using **PDP** coordinates (**PDP** ID. 1DAR).

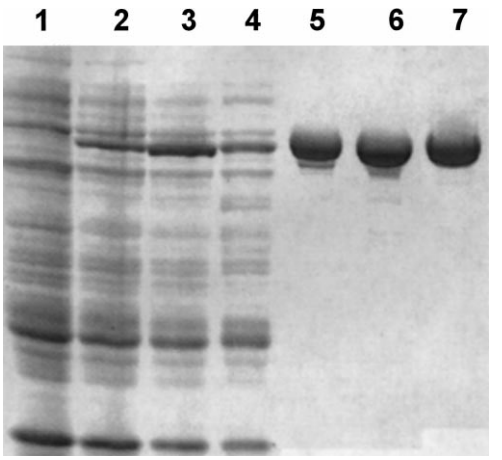

Fig. 2. SDS electrophoresis. Lane 1: cell extract without plasmid; lanes 2–4: crude extracts with plasmids carrying *fus* genes with H753A, H573/D576A and insertion mutations, respectively; lanes 5–7: purified mutated proteins (given in the same order as the mutated genes).

T. thermophilus EF-G is active in a cell-free translation system with ribosomes from *E. coli* [10], therefore functional tests were carried out in vitro with *E. coli* components. All mutated factors possessed the same ribosome-dependent GTPase activity as wild type EF-G (about 30 nmol of GTP was hydrolyzed for 10 min in 100 μl of the reaction mixture).

The proteins with double and H573A mutations stimulated poly(U)-directed translation in a way similar to the wild type EF-G (Fig. 3A). This fact suggests that residues H573 and D576 per se are not important for the translocation activity of EF-G, i.e. their side chains do not participate in any exclusive contacts with ribosomal components.

On the other hand, the functional activity of the EF-G with the insert was decreased markedly in poly(U)-directed translation (Fig. 3B). The puromycin reaction promoted by the mutated factor was greatly decreased as well (Fig. 4). It is reasonable to assume that six amino acids inserted into the 573–579 loop can change the conformation of the loop. These data provide direct evidence that intact conformation at the tip of EF-G domain IV is necessary for flawless interaction with ribosomal components or peptidyl tRNA.

Hamster elongation factor contains the His-715 residue [16] which is modified post-translationally to diphthamide. This unusual amino acid is specifically ADP-ribosylated by diphtheria toxin and this modification inactivates EF-2. The amino acid of bacterial EF-G corresponding to His-715 of mammalian EF-2 could then also be functionally important [2,4–6]. Despite some ambiguity in the sequence alignment [15,16] amino acids corresponding to EF-2 are found in the sequence fragment 573–578 of *T. thermophilus* EF-G. According to this His-573 and D576 of EF-G correspond to H710 and H715 of EF-2, respectively. However, our results show that the replacement of residues H573 and D576 by alanine did not lead to inactivation of the factor, but the peptide insertion did. These results suggest that the size and the native conformation of the loop are generally more important than its

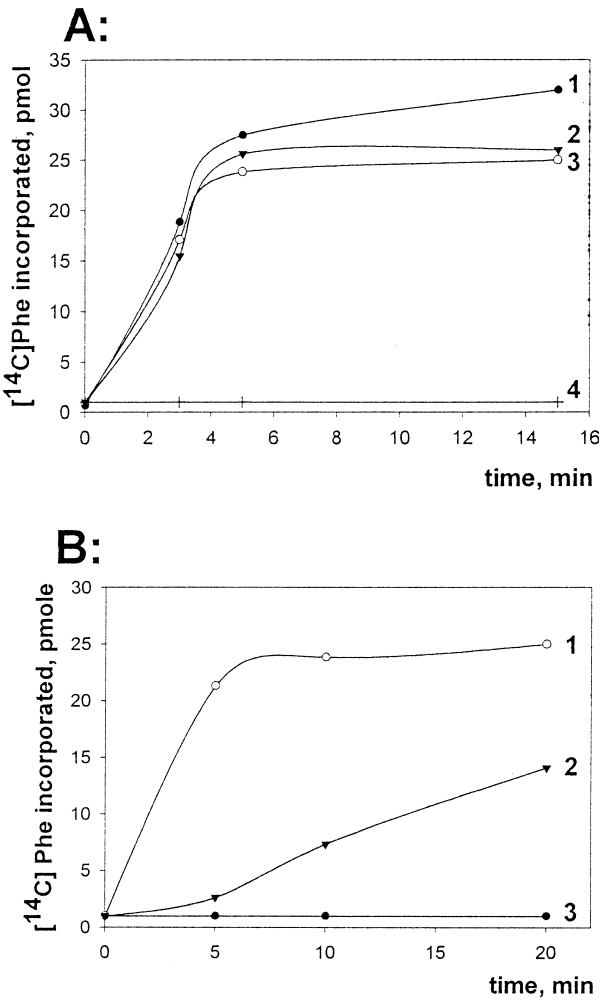

Fig. 3. Kinetics of poly(U)-directed cell-free translation. A: 1: H573A mutated EF-G added; 2: EF-G with H573/D576 mutation; 3: wild type EF-G; 4: no EF-G added. B: 1: wild type EF-G; 2: EF-G with the insert; 3: no EF-G added.

amino acid composition. It should be noted that point mutations introducing residues with bulky or negatively charged side chains can influence the loop conformation as well as its interactions with the ribosomal components. The ADP-ribosylation and some point mutations of EF-2 could lead to similar consequences. For example, His-715 replacement of hamster EF-2 by Asp, Lys or Arg impairs its function [17], but replacement of the corresponding His-699 of yeast EF-2 by Asn, Gln, Leu or Met does not.

Release factors (RF) are needed for termination of the translation. RF-1 and RF-2 interact specifically with the stop codons in the ribosomal decoding site and are thought to mimic domain IV and partially domain III of EF-G [18]. It is interesting that the release factors do not contain the residues YHDV which are present in the YHEVDS loop (residues 572–577) of *T. thermophilus* EF-G and are highly homologous in bacterial EF-G. However, the release factors have a sequence identical to the SGGR motif of *E. coli* EF-G [19]. The homologous loop TGGR (residues 501–504) of *T. thermophilus* EF-G connects the β2/β3 strands and is spatially very close to the loop YHEVDS (Fig. 1). Both these loops build up the distal end (tip) of EF-G domain IV [5,6]. This

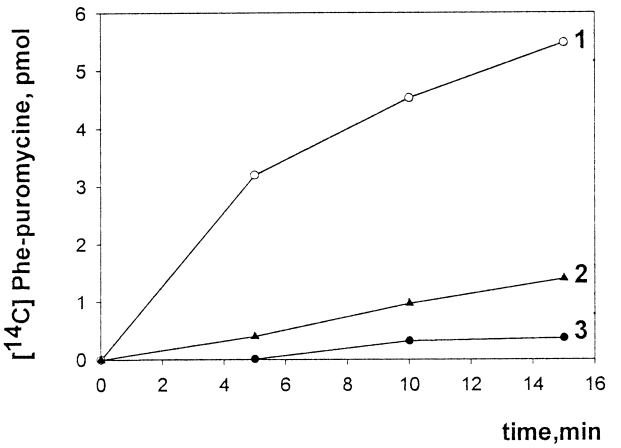

Fig. 4. Kinetics of puromycin reaction with pre-translocation ribosomes. 1: reaction was promoted by wild type EF-G; 2: EF-G with the insert; 3: no EF-G added.

observation may suggest a functional relationship of these factors and the importance of the tip of domain IV as a whole.

On the basis of the results cited above it can be concluded that an intact conformation of the YHEVDS loop of domain IV is important for translocation. We propose also that the entire tip of domain IV is important for interactions with components at the decoding site of the ribosome.

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