Role of the conserved aspartate and phenylalanine residues in prokaryotic and mitochondrial elongation factor Ts in guanine nucleotide exchange

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Abstract The guanine nucleotide exchange reaction catalyzed by elongation factor Ts is proposed to arise from the intrusion of the side chains of D80 and F81 near the Mg^{2+} binding site in EF-Tu. D80A and F81A mutants of *E. coli* EF-Ts were 2–3-fold less active in promoting GDP exchange with *E. coli* EF-Tu while the D80AF81A mutant was nearly 10-fold less active. The D84 and F85 mutants of EF-Ts$_{mt}$ were 5–10-fold less active in stimulating the activity of EF-Tu$_{mt}$. The double mutation completely abolished the activity of EF-Ts$_{mt}$.

Key words: Protein synthesis; Elongation factor; Mitochondria; Elongation factor Tu; Elongation factor Ts

1. Introduction

During the process of protein biosynthesis, EF-Tu promotes the binding of aminoacyl-tRNA (aa-tRNA) to the A-site of the ribosome [1]. This reaction is mediated through a ternary complex [EF-Tu:GTP:aa-tRNA]. Following ribosome binding, the GTP is hydrolyzed and EF-Tu is released from the ribosome as an EF-Tu:GDP complex. GDP is then exchanged for GTP in a process mediated by EF-Ts [2]. The structure of EF-Tu is known in both the GDP- and GTP-bound forms [3,4]. This protein folds into three domains. Domain I, encompassing the first 200 residues, contains the guanine nucleotide binding site, while all three domains are involved in binding aa-tRNA [5]. The structure of the *E. coli* EF-Tu·Ts complex has recently been determined [6]. Examination of this structure suggests that nucleotide exchange arises in part because the side chains of D80 and F81 of EF-Ts intrude near the site on EF-Tu where the Mg^{2+} ion interacting with GDP is normally located. The resulting disruption of the Mg^{2+} ion binding site is believed to reduce the affinity of EF-Tu for GDP.

The mammalian mitochondrial factors equivalent to EF-Tu and EF-Ts have been purified from bovine liver mitochondria as a tightly associated complex (EF-Tu·Ts$_{mt}$) [7,8]. This complex, unlike the corresponding complex from *E. coli*, is not dissociated by either GDP or GTP even at high concentrations of the guanine nucleotides. The cDNAs for both of these proteins have been cloned and sequenced [9,10]. EF-Tu$_{mt}$ has 55–60% identity to prokaryotic EF-Tu. In contrast, EF-Ts$_{mt}$ is less than 30% identical to the bacterial factors. Mammalian EF-Ts$_{mt}$ has residues corresponding to D80 and F81 postulated to be involved in the guanine nucleotide exchange reac-

tion with *E. coli* EF-Ts. When EF-Ts$_{mt}$ is expressed in *E. coli*, it forms a tight complex with *E. coli* EF-Tu [10]. The properties of this heterologous complex are similar to those of the mitochondrial EF-Tu·Ts$_{mt}$ complex and, unlike the *E. coli* EF-Tu·Ts complex, the heterologous complex formed with EF-Ts$_{mt}$ is not dissociated detectably by guanine nucleotides. These observations suggest that EF-Ts$_{mt}$ confers several unusual properties upon the EF-Tu·Ts complex. In the current manuscript, we have examined the roles of the conserved Asp and Phe residues in both *E. coli* EF-Ts and EF-Ts$_{mt}$ that are thought to facilitate the guanine nucleotide exchange reaction.

2. Materials and methods

2.1. Construction of clones and mutants
The *E. coli* EF-Tu and EF-Ts genes were amplified from *E. coli* chromosomal DNA by PCR and cloned into pET24C(+) (Novogen). This vector provides a His-tag to facilitate purification of the expressed proteins on Ni-NTA resins. Site-directed mutagenesis of the *E. coli* EF-Ts and EF-Ts$_{mt}$ genes was performed using a PCR-based 'link scanning' method [11].

2.2. Expression and purification of EF-Tu and EF-Ts
The His-tagged form of *E. coli* EF-Tu was expressed and purified as described previously [9] except that 10 mM MgCl$_2$ and 10 μM GDP were included. *E. coli* EF-Ts was purified under three different conditions. (1) Extracts were prepared as described [9] in buffer containing MgCl$_2$; (2) 10 μM GDP was also included in the isolation buffer. This condition was used for the large-scale preparation of EF-Ts and its mutants; (3) no MgCl$_2$ was added to the extraction buffers and the cell extract was dialyzed twice against a 100-fold excess of buffer containing 10 mM Tris-HCl, pH 7.6, 40 mM KCl and 10% glycerol prior to purification of EF-Ts. Expression of EF-Ts$_{mt}$ was carried out as described [10]. When cell extracts were prepared under native conditions, EF-Ts$_{mt}$ was isolated as a 1:1 complex with *E. coli* EF-Tu (EF-Tu$_{Eco}$·Ts$_{mt}$). To purify EF-Ts$_{mt}$ free of *E. coli* EF-Tu, the EF-Tu$_{Eco}$·Ts$_{mt}$ complexes were denatured. EF-Ts$_{mt}$ was then purified through a Ni-NTA column and renatured (Xin and Spremulli, in preparation).

2.3. Assays
The activities of *E. coli* EF-Ts and its mutated forms were determined by measuring their ability to promote guanine nucleotide exchange with *E. coli* EF-Tu:GDP and to stimulate the activity of EF-Tu in the poly(U)-directed polymerization of phenylalanine on *E. coli* ribosomes [12,13]. The activity of EF-Ts$_{mt}$ was determined by the ability to stimulate the activity of EF-Tu$_{mt}$ (Xin and Spremulli, in preparation).

3. Results and discussion

3.1. Conserved D and F residues in E. coli EF-Ts
Three mutants of *E. coli* EF-Ts (D80A, F81A and D80AF81A) were made by site-directed mutagenesis. When

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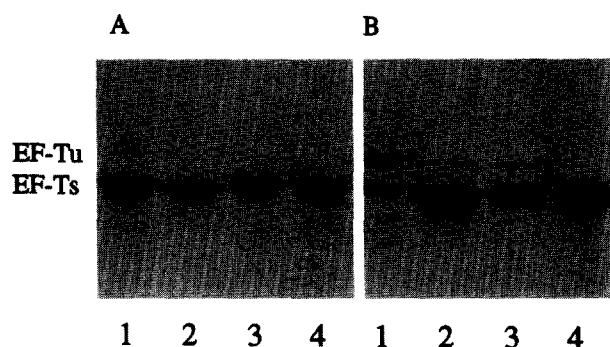
A   B

Fig. 1. SDS-PAGE analysis of the expressed *E. coli* EF-Ts and its mutants. (A) *E. coli* EF-Ts and its mutants were purified using method (1). Lanes: 1, wild-type; 2, D80A; 3, F81A; 4, D80AF81A. (B) Extracts were prepared using method (3). Lanes: 1, wild type; 2, D80A; 3, F81A; 4, D80AF81A.

a His-tagged derivative of wild-type EF-Ts is prepared from *E. coli* in buffer containing Mg^{2+} and purified on Ni-NTA resin, a small amount of EF-Tu can be observed in the purified preparation (Fig. 1A, lane 1). This observation suggests that most of the EF-Tu in the extract is complexed with GDP or in the ternary complex. Under similar conditions, no EF-Tu could be observed associated with the D80A, F81A or the D80AF81A mutated forms of EF-Ts (Fig. 1A, lanes 2–4). This observation suggests that the mutation of the conserved D and F residues reduces the affinity of EF-Ts for EF-Tu
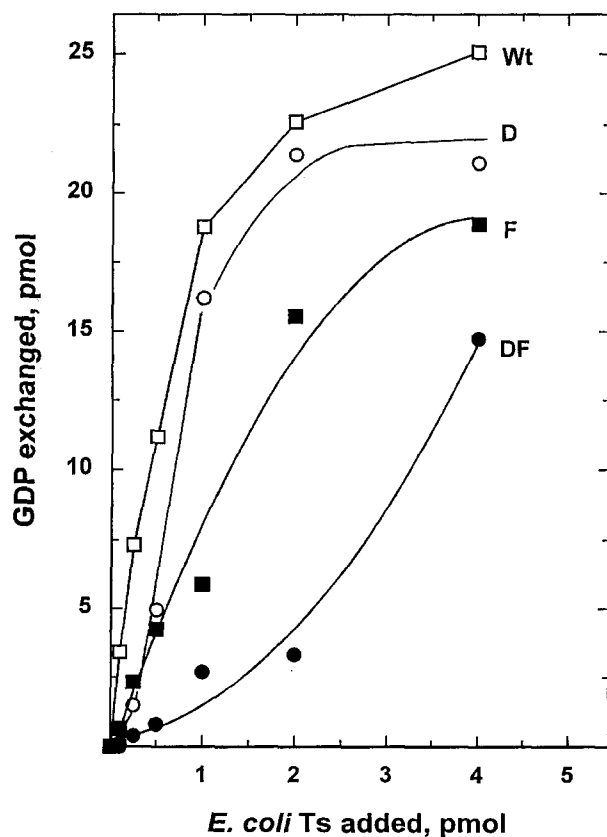

Fig. 2. Stimulation of GDP exchange by *E. coli* EF-Ts and its mutants. Nucleotide binding assays contained 3.6 μg of expressed *E. coli* EF-Tu and the indicated amount of wild-type or mutated forms of *E. coli* EF-Ts. Wt, wild-type; D, D80A; F, F81A; DF, D80AF81A. Incubation was at 0°C for 5 min.
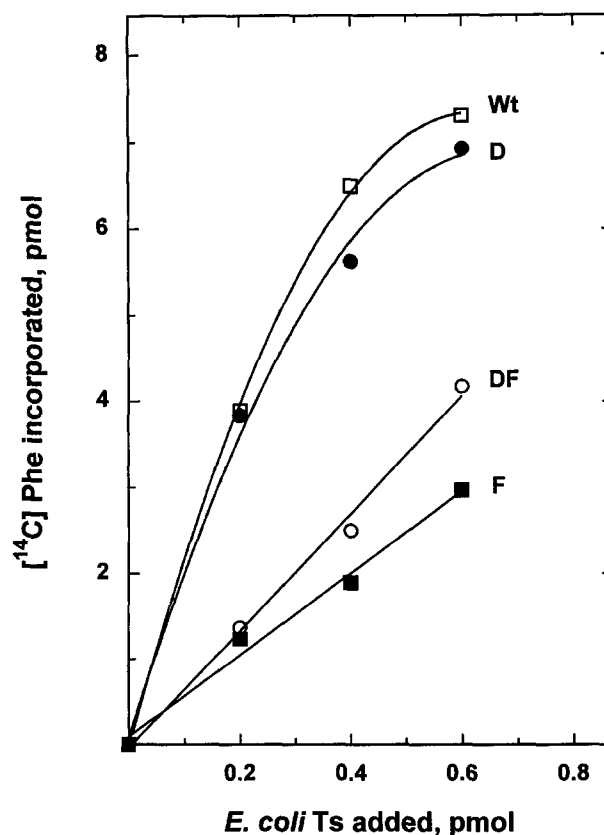

Fig. 3. Stimulation of *E. coli* EF-Tu in poly(U)-directed polymerization: Reaction mixtures contained 0.75 pmol of *E. coli* EF-Tu and the indicated level of *E. coli* EF-Ts. Wt, wild-type; D, D80A; F, F81A; DF, D80AF81A.

significantly. When extracts were prepared in buffers lacking Mg^{2+} and dialyzed prior to purification on Ni-NTA, wild-type EF-Ts is purified as a 1:1 complex with EF-Tu (Fig. 1B, lane 1). In contrast, the ratio of *E. coli* EF-Tu to the D80A and F81A derivatives of EF-Ts is less than 1:10 (Fig. 1B, lanes 2,3). The ratio of *E. coli* EF-Tu to the D80AF81A form of EF-Ts is even lower and EF-Tu could barely be detected in these preparations (Fig. 1B, lane 4). These data again suggest that all three mutated derivatives of *E. coli* EF-Ts can interact with *E. coli* EF-Tu, but that the interactions are much weaker than that observed with wild-type EF-Ts.

EF-Ts is very active in stimulating nucleotide exchange with *E. coli* EF-Tu:GDP (Fig. 2). The D80A mutant has about 2-fold lower activity than the wild-type EF-Ts at limiting concentrations of EF-Ts while the F81A mutant has about 3-fold lower activity. The double mutant shows a somewhat sigmoidal response characteristic of a defect in the interaction of two proteins in a 2-component system. At lower concentrations, it is about 10-fold less active than the wild-type EF-Ts but has significant activity at higher concentrations. These observations suggest that both D80 and F81 are important but not essential for the function of *E. coli* EF-Ts.

The D80A mutant is almost as active as wild-type EF-Ts in stimulating the poly(U)-directed polymerization of phenylalanine (Fig. 3). The F81A and the double mutant are about 4-fold less active than wild-type EF-Ts in this assay. The polymerization assay appears to be somewhat less sensitive than the GDP-exchange assay. The availability of aa-tRNA in this assay most likely pulls the nucleotide exchange reaction in the
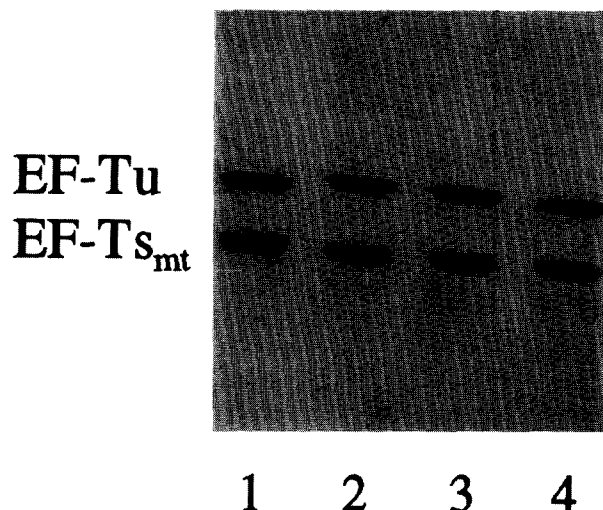
Fig. 4. SDS-PAGE analysis of purified EF-Tu$_{Eco}$·Ts$_{mt}$ complexes. Extracts were prepared under native conditions from cells expressing wild-type and mutated forms of EF-Ts$_{mt}$. Lanes: 1, wild-type EF-Ts$_{mt}$; 2, D84A; 3, F85A; 4, D84AF85A.

forward direction by the formation of the ternary complex and subsequent ribosome binding. Hence, the reduced ability of the mutated forms of EF-Ts to promote guanine nucleotide exchange may not be as apparent when coupled to the favorable formation of the ternary complex.

3.2. Role of D84 and F85 in EF-Ts$_{mt}$

The corresponding mutants (D84A, F85A and D84AF85A) were prepared in bovine EF-Ts$_{mt}$. When wild-type EF-Ts$_{mt}$ is prepared from *E. coli* under non-denaturing conditions, it is present as a 1:1 complex with *E. coli* EF-Tu (Fig. 4, lane 1 and [10]). All three of the mutated forms of EF-Ts$_{mt}$ are able to form tight complexes with *E. coli* EF-Tu (Fig. 4, lanes 2–4). This observation indicates that neither D84 nor F85 plays a crucial role in allowing the interaction of EF-Ts$_{mt}$ with EF-Tu. This observation is in contrast with those made with *E. coli* EF-Ts where mutation of either of these residues significantly weakens the interaction between EF-Tu and EF-Ts.

The ability of EF-Ts$_{mt}$ to stimulate GDP exchange with EF-Tu$_{mt}$ cannot be tested directly since no direct GDP binding to this factor can be demonstrated. The ability of wild-type EF-Ts$_{mt}$ and its mutated derivatives to stimulate the activity of EF-Tu$_{mt}$ in poly(U)-directed polymerization was, therefore, tested. Both D84A and F85A derivatives of EF-Ts$_{mt}$ show significantly lower activity than wild-type EF-Ts$_{mt}$ (Fig. 5). The dose responses are sigmoidal, suggesting some defect in the interaction between these two proteins that was not apparent by the SDS-PAGE analysis. The D84AF85A variant is completely inactive. The combination of these residues appears to play a more essential role in EF-Ts$_{mt}$ than in *E. coli* EF-Ts.
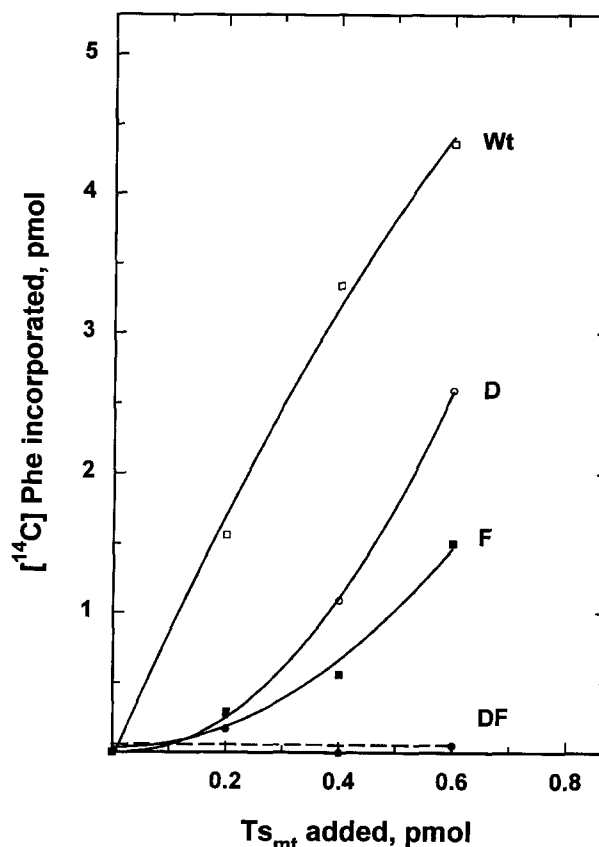

Fig. 5. Stimulation of the activity of EF-Tu$_{mt}$ in poly(U)-directed polymerization: reaction mixtures contained 2 pmol of EF-Tu$_{mt}$ and the indicated amount of EF-Ts$_{mt}$. Wt, wild-type; D, D84A; F, F85A; DF D84AF85A.

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