Protein engineering on enzymes of the peptide elongation cycle in *Sulfolobus solfataricus*

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Abstract — The present article is a review of the work done on the elongation factors EF-1α, EF-2 and EF-1β isolated from the hyperthermophilic archaeon *Sulfolobus solfataricus*. The molecular, physical and biochemical properties of the intact, truncated, mutant or chimeric forms are described and compared. © Société française de biochimie et biologie moléculaire / Elsevier, Paris

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1. Introduction

In the process of protein synthesis the elongation of the growing peptide chain is governed in all the living organisms by three protein factors (for a review see [1]). In the archaea the elongation factor 1α (EF-1α) bound to GTP is the carrier of the aminoacyl-tRNA (aa-tRNA) to the mRNA•ribosome complex. Following the interaction of the EF-1α•GTP•aa-tRNA complex with the heavier ribosomal subunit, the GTP is hydrolyzed and the inactive EF-1α•GDP complex moves away from the ribosome leaving the aa-tRNA on the ribosomal A site. The formation of a new peptide bond between the aa-tRNA and the peptidyl-tRNA, which is located on the P site of the ribosome, is catalyzed by the peptidyl-transferase. The elongation factor 2 (EF-2) bound to GTP translocates the newly formed peptidyl-tRNA from the A site to the P site of the ribosome; then the A site becomes available for a next cycle. The exchange factor 1β (EF-1β) converts EF-1α•GDP into EF-1α•GTP which is able to bind a molecule of aa-tRNA, thus allowing the start of a new cycle.

From this synopsis it appears that in the fulfillment of their biological functions the enzymes of the elongation cycle interact with guanine nucleotides, aminoacyl-tRNA, ribosome and other proteins; in addition, two of them, EF-1α and EF-2, are GTP binding proteins [2] and following their interaction with the ribosome they elicit a GTPase activity in two crucial steps of the elongation cycle, i.e., the binding of the aa-tRNA to the ribosome and the translocation reaction. Therefore, both EF-1α and EF-2 are multifunctional proteins and then excellent

models for protein engineering; in fact, the effects of any modification (including mutations or deletions) can be evaluated by several physical or biochemical tools. In addition, EF-1α and EF-2 isolated from thermophilic organisms are endowed with a great resistance against heat [1] and therefore they are suitable models to study the molecular bases of thermostability.

The present article describes in brief the work done during the last years in this laboratory on the elongation factors SsEF-1α, SsEF-2 and SsEF-1β in the hyperthermophilic archaeon *Sulfolobus solfataricus* (growth conditions: 87 °C, pH 3.5). SsEF-1α and SsEF-2 promote poly(Phe) synthesis, bind GDP and GTP [3–5] and display an intrinsic GTPase activity which is triggered in vitro by NaCl at molar concentrations (GTPase^a) or by ethylene glycol (GTPase^g) respectively [6, 7]; furthermore, SsEF-2 elicits a GTPase activity in the presence of ribosome (GTPase^r) [8]. SsEF-1β has been proved to accelerate the GDP/GTP exchange on SsEF-1α•GDP [9]. Mutated or deleted forms of all three of them have been produced by gene engineering, expressed in *E. coli*, purified, characterized and their molecular, physical and biochemical properties have been compared with those of the native proteins [10, 11].

2. Properties of truncated forms of SsEF-1α

SsEF-1α is a monomeric protein with a M_r 49 000; its isoelectric point is 9.1. It is a GTP binding protein endowed with a remarkable thermophilicity (the rate of the GDP binding reaches a maximum at 95 °C) and it is very resistant to heat (after 6 h exposure at 87 °C its GDP binding capacity is still 54% of the control) [12].

Two truncated forms of SsEF-1α have been produced [10]: one, Ss(G), constituted by the nucleotide

Table I. Affinity for GDP and GTP of the chimera Ss-(G)Ec(MC) and of the truncated forms Ss(G) and Ss(GM) of SsEF-1α. Comparison with intact SsEF-1α and EcEF-Tu.

Elongation factor	Temperature (°C)	K_d (µM) GDP	GTP	Reference
Ss(G)Ec(MC)	60	0.6	5.6	This work
Ss(G)	60	0.2	1.7	[10]
Ss(GM)	60	0.4	7.4	[10]
SsEF-1α	60	1.6	35	[10]
EcEF-Tu	0	0.0009	0.5	[13]

Table II. Heat stability of the chimera Ss(G)Ec(MC) and of the truncated forms Ss(G) and Ss(GM) of SsEF-1α. Comparison with intact SsEF-1α and EcEF-Tu.

| Elongation factor	Temperature for half inactivation (°C)	Reference
Ss(G)Ec(MC)	81	This work
Ss(G)	84	[10]
Ss(GM)	84	[10]
SsEF-1α	94	[10]
EcEF-Tu	51	[10]

The temperature for half inactivation was evaluated by the residual GDP binding ability of each protein after 10 min of treatment in proper temperature ranges.

binding domain G, and the other Ss(GM) containing the middle- (M) and the G-domain. Ss(G) and Ss(GM) were unable to sustain poly(Phe) synthesis; therefore, it seems that the remaining domain C is essential for protein synthesis. However, Ss(G) and Ss(GM) were still able to bind GDP and GTP even with an increased affinity compared to the intact SsEF-1α: the values of the equilibrium dissociation constant K_d for GDP referred to SsEF-1α, Ss(G) and Ss(GM) were 1.6, 0.2 and 0.4 µM respectively; the corresponding values for GTP were estimated as 35, 1.7 and 7.4 µM *(table I)*. The GTPase* activity of SsEF-1α was not affected by the deletions since the values of k_{cat} and K_m remained unchanged. Vice versa, the thermostability of both Ss(G) and Ss(GM) was significantly reduced, being the temperature for half inactivation 84 °C, a value 10 °C below that of the intact form. The thermophilicity of Ss(GM) was the same as that of the intact SsEF-1α, whereas Ss(G) was definitely less thermophilic. Therefore, it seems that the domain M controls the thermophilicity of SsEF-1α. Both Ss(G) and Ss(GM) showed a [³H]GDP/GTP exchange rate that was much faster compared to that of intact SsEF-1α and not further stimulated by SsEF-1β. This finding suggested that a putative site of the interaction between intact SsEF-1α and SsEF-1β is located on the C domain. However, since the nucleotide exchange on the isolated G and GM domains no longer required the stimulation by SsEF-1β, it may be inferred that the failure of Ss(G) and Ss(GM) to support the synthesis of poly(Phe) was not due to the lack of interaction of the domain G with SsEF-1β.

3. Properties of a chimeric EF-1α constituted by the nucleotide binding domain of *Sulfolobus solfataricus* EF-1α and the *Escherichia coli* EF-Tu MC domain

Gene engineering has been used to construct a chimeric EF-1α made of the hyperthermophilic domain Ss(G) (M_r 26 200) and the mesophilic domain MC (M_r 20 800) from *Escherichia coli* (Ec(MC)). The chimera Ss(G)Ec(MC) showed an affinity for GDP and GTP, estimated by the equilibrium dissociation constant K_d, which was about three and six times higher respectively compared to

SsEF-1α *(table I)*. In the case of GDP this was due to the fact that the rate of association of the complex Ss(G)Ec(MC)–GDP increased about three times more than the rate of dissociation. Regarding its intrinsic NaCl triggered GTPase* the chimera showed a three-fold increase of the catalytic efficiency. Ss(G)Ec(MC) was more thermophilic than SsEF-1α up to 70 °C; the inactivation observed at higher temperatures was very likely due to the thermal inactivation of the mesophilic moiety of the chimera. The decreased thermostability was confirmed by the thermal inactivation profiles of EcEF-Tu, Ss-(G)Ec(MC) and SsEF-1α evaluated by GDP binding. In fact, the half life of the chimera was 10 min at 81 °C, a temperature 13 °C lower than that of SsEF-1α but 30 °C higher than that determined for EcEF-Tu *(table II)*. These results indicated that the thermostability of the chimera was not greatly impaired by the mesophilic Ec(MC) domains.

4. Mutant analysis of SsEF-2

The translocation enzyme SsEF-2 is a GTP binding protein made of 735 amino acid residues [14]. At 87 °C, the growth temperature of *S. solfataricus*, its half-life was 3 h [5]. Once ADP-ribosylated by diphteria toxin in the presence of NAD the native SsEF-2 is no longer able to sustain poly(Phe) synthesis in vitro [4]. The mutant analysis SsEF-2 regarded one of the three consensus sequences [G,A]XXXXGK, DXXG and NKXD involved in the binding of GDP or GTP [2]. In the [G,A]XXXXGK sequence the first amino acid residue is G in EF-Tu's and EF-1α's, whereas it is A in all the EF-G's and EF-2's. In the attempt to clarify the functional significance of such a structural difference, the A/G mutation was introduced by site directed mutagenesis at position 26 of SsEF-2, which corresponds to the first position of the AXXXXGK sequence. The Ss(A26G)EF-2 mutant has been produced in *E. coli*, purified and characterized. In a system contain-
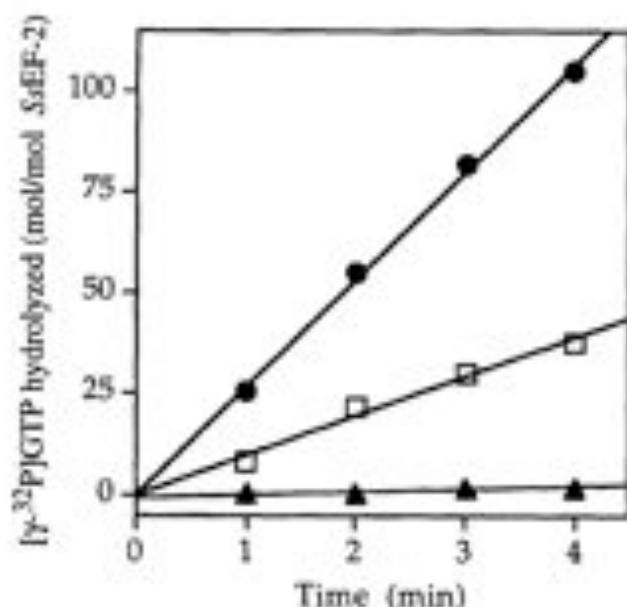
Figure 1. Kinetics of the ribosome-dependent GTPase of SsEF-2 and its engineered forms. The GTPase activity was assayed at 60 °C in a reaction mixture containing 0.1 μM SsEF-2 (●), Ss(A26G)EF-2 (□) or Ss(Δ124C)EF-2 (▲). The details are reported in [8].

ing all the other components required for protein synthesis, the substitution of SsEF-2 for Ss(A26G)EF-2 produced a moderate reduction of the synthesis of poly(Phe), which could be attributed to a slower turnover of the ribosome dependent GTPase of Ss(A26G)EF-2 compared to that determined for SsEF-2 (figure 1). On the other hand, the activity of the intrinsic GTPase of Ss(A26G)EF-2 was significantly high, even in the absence of ethylene glycol. All these results might suggest that A26 regulates the GTPase activity of SsEF-2.

5. Properties of SsEF-2 truncated at the C-terminal end

The 3D structure of SsEF-2 is not available yet and therefore the structural domains of SsEF-2 have been putatively identified from the alignment of the amino acid sequence of SsEF-2 with that of *Thermus thermophilus* EF-G, whose 3D structure has been solved [15, 16]. Gene engineering was used to construct a truncated form of SsEF-2, Ss(Δ124C)EF-2, lacking the final 124 amino acid residues; this segment corresponded essentially to a putative region required for the interaction of SsEF-2 with the ribosome. Ss(Δ124C)EF-2 showed a reduced thermosta-

bility, being the temperature for half-inactivation 89 °C that was 7 °C lower than that of intact SsEF-2. Ss(Δ124C)EF-2 was unable to sustain poly(Phe) synthesis. This finding was due to the lack of the peptide region required for the interaction of SsEF-2 with the ribosome; such a hypothesis was supported by the failure of Ss(Δ124C)EF-2 to display the ribosome dependent GTPase activity (figure 1), whereas its intrinsic GTPase is maintained.

6. Mutant analysis of SsEF-1β

The exchange factor SsEF-1β is made of two identical subunits [9], each one containing 90 amino acid residues [17]. Its primary structure is homologous to the C-terminal region of eucarial EF-1β that contains the site of interaction with SsEF-1α [9]. The purified factor accelerated the rate of [³H]GDP/GTP exchange on the SsEF-1α·GDP complex, while, as already mentioned, no effect was detectable on the complexes formed between GDP and Ss(G) and Ss(GM) [10]. This finding suggested that the domain C of SsEF-1α is involved in the interaction with EF-1β. The Ss(Y54H)EF-1β mutant, carrying a bulkier residue in the hydrophobic core of the molecule, displayed biochemical properties indistinguishable from those of native SsEF-1β [11]. Both the native and the mutant forms were 50% inactivated after 30 min of treatment at 105 °C.

7. Concluding remarks

From the data reported above it appears that the assay of biological properties combined with protein engineering can be useful to investigate whether structural and functional resemblance exists between homologous proteins.

The observation that in the absence of ribosome the unmasking of the intrinsic hardly detectable GTPase of SsEF-1α and SsEF-2 requires a chemical environment similar to that described for EcEF-Tu and EcEF-G respectively, suggests that structural similarities exist between EcEF-Tu and SsEF-1α and between EcEF-G and SsEF-2. In particular, high salt concentrations are needed for SsEF-1α [6] and EcEF-Tu [18] and aliphatic alcohols for SsEF-2 [7] and EcEF-G [19].

The resistance of SsEF-1α and SsEF-2 against thermal inactivation required the integrity of the entire molecule, since compared to the intact factors the truncated forms of either SsEF-1α or SsEF-2 showed a significantly reduced thermostability. The molecular integrity of SsEF-1α and SsEF-2 was also important for the synchronous elicitation of their respective biochemical properties. In fact, all the truncated forms were unable to sustain protein synthesis in vitro but they still maintained their ability to bind and

hydrolyze GTP. Finally, it is worthy noting that the effects produced on the archaeal factors by truncations were predictable from the alignment with the respective eubacterial counterparts. Therefore, it can be inferred that the overall molecular organization of homologous elongation factors is similar.

Designed amino acid replacement will allow a deeper insight on the functional and structural properties of *S. solfataricus* elongation factors.

Acknowledgments

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