Biochimie 91 (2009) 835–842

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

Biochemical characterisation of the D60A mutant of the elongation factor 1α from the archaeon *Sulfolobus solfataricus*

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ARTICLE INFO

Article history:
Received 5 March 2009
Accepted 2 April 2009
Available online 16 April 2009

Keywords:
Elongation factor 1α
Mutagenic analysis
Nucleotide binding site
Heat stability
Sulfolobus solfataricus

ABSTRACT

The D60A mutant of the elongation factor (EF) 1α from *Sulfolobus solfataricus* (Ss), was obtained as heterologous expressed protein and characterised. This substitution was carried out in order to analyse the involvement of this evolutionally conserved amino acid position in the interaction between the elongation factor and guanosine nucleotides and in the coordination of magnesium ions. The expression system used produced a folded protein able to catalyse, although to a slightly lower extent with respect to the wild-type enzyme, protein synthesis *in vitro* and NaCl-dependent intrinsic GTPase activity. The affinity for guanosine nucleotides was almost identical to that exhibited by wild-type SsEF-1α; vice versa, the GDP exchange rate was one order of magnitude faster on the mutated elongation factor, a property partially restored when the exchange reaction was analysed in the presence of the magnesium ions chelating agent EDTA. Finally, the D60A substitution only a little affected the high thermal stability of the elongation factor. From a structural point of view, the analysis of the data reported confirmed that this conserved carboxyl group belongs to a protein region differentiating the GDP binding mode among elongation factors from different organisms.

1. Introduction

The process of protein biosynthesis is central in cell life [1]. In addition to ribosome, efficient and reliable translation of the information stored in mRNA to protein sequences requires the intervention of several protein factors [2,3]. Among these, a fundamental role is played by a family of GTPase enzymes denoted as translational elongation factors (EF) that promote the binding of the aminoacyl-tRNA to ribosome. EFs are universal proteins present in all three domains of life [2,4]. EFs isolated from different organisms display analogies and differences. They share a significant sequence identity (higher than 30%), with eukaryal and archaeal EFs (EF-1α) exhibiting higher similarities (sequence identity higher than 50%). The sequence identities of eubacterial EF (EF-Tu) with their eukaryal and archaeal counterparts fall in the range of 30–35% [5]. From the structural point of view, EF-1αs and EF-Tus share important general features: the overall organization in three distinct domains and a high plasticity in response to the interactions with their biological partners [6–15].

Thermodynamics, structural, and biochemical investigations on EFs isolated from different sources have also highlighted striking differences. A significant correlation of the EF thermal stability with the living conditions of the host organism has been discovered [16]. In their ligand-free states, EFs isolated from mesophilic and thermophilic eubacteria, exhibit non-cooperative multi-state unfolding; however, the cooperativity of the unfolding process of these EFs increases upon the addition of ligands such as GDP or GTP analogs [17,18]. On the other hand, EF-1α extracted from the hyperthermophilic archaeon *Sulfolobus solfataricus* (SsEF-1α) shows a cooperative unfolding upon chemical or thermal denaturation regardless of its binding state [19,20]. These differences likely reflect dissimilar inter-domain interactions in the various members of this multi-domain protein family. Crystallographic studies have demonstrated that eukaryal and eubacterial EFs adopt completely different molecular recognition modes to interact with

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doi:10.1016/j.biochi.2009.04.003

their exchange factors partners [10–13], which are needed for the GDP release. Finally, biochemical investigations have unveiled that magnesium ion plays quite different roles in the modulation of the activity of eubacterial, archaeal and eukaryal EFs. Indeed, the characterisation of these enzymes has indicated that the ion is involved in different stages of the EF action (nucleotide binding or GTP hydrolysis). In eubacterial EF-Tus, Mg^{2+} is essential for the nucleotide binding, whereas it does not play an important role in the GTP hydrolysis [21]. Particularly puzzling is the observation that the reverse is observed for the archaeal SsEF-1α for which the cation is not required for nucleotide binding, whereas it is essential for its intrinsic GTPase activity [14,22]. Differences on the role played by the ion in archaeal or eukaryal EF-1αs are also emerging [14,23]. Indeed, it has been reported that in *Saccharomyces cerevisiae* EF-1α the Mg^{2+} induces a significant decrease of GDP exchange reaction catalysed by the exchange factor EF-1β [23].

In order to provide further data on the role that Mg^{2+} plays in different members of this protein family, we have analysed the involvement of the residue Asp60 of SsEF-1α in its biochemical properties through a mutagenic analysis. In fact, it is expected that this conserved residue should play different roles in archaeal and eubacterial factors. Indeed previous structural studies have indicated that this residue is not engaged in magnesium coordination in the binary complex SsEF-1α·GDP [14], whereas the corresponding residue (Asp50) is involved in the Mg^{2+} binding in the complex of EF-Tu with GDP [6,24]. The analysis of the available three-dimensional structural models of SsEF-1α provides clear indications of the structural basis of the alteration produced by the mutation on the enzyme activity.

2. Materials and methods

2.1. Materials

Restriction enzymes, modifying enzymes, labelled compounds, and chemicals were used as already reported [25]. Except otherwise indicated, all DNA manipulation were carried out according to standard methodologies [26]. The procedure for the construction of the heterologous expression vector and for the purification of wild-type SsEF-1α was as already reported [27]. GppNHp was from Sigma–Aldrich and was further purified by anion exchange chromatography on MonoQ (Pharmacia). The binary complex between the elongation factors and GDP or GppNHp were formed as reported [28].

The following buffers were used: buffer A, 20 mM Tris–HCl, pH 7.8, 50 mM KCl, and 10 mM $MgCl_2$; buffer B, 20 mM Tris–HCl, pH 7.8, 10 mM $MgCl_2$, and 1 mM DTT.

2.2. Plasmid construction, expression, and purification of the mutant elongation factor

Site-directed mutagenesis was carried out using a commercial kit from Stratagene and the pT7-7 derivative cloning vector used for the expression of wild-type SsEF-1α [27] as template in a PCR reaction. We used two self-complementary primers for each mutation planned, namely C_{171}CTACTAGCTAGATTAAAAGAG (D60A forward) and CTCTTTTAATCTAGCTAGTAGG (D60A reverse) or C_{171}CTACTAGAAAGATTAAAAGAG (D60E forward) and CTCTTTTA ATCTTTCTAGTAGG (D60E reverse), carrying the $A_{179}\rightarrow C$ or $T_{180}\rightarrow A$ base substitutions, as numbered from the EF-1α starting codon, to obtain the D60→A or D60→E amino acid substitution in SsEF-1α. The products of the PCR obtained were then used to transform XL11 Epicurian competent cells and the recombinant plasmids were analysed for nucleotide sequence to assess the base substitutions. The new plasmids obtained (D60AvEF-1 and D60EvEF-1) were used to transform *Escherichia coli* BL21(DE3) competent cells. An overnight cell culture grown at 37 °C in LB-broth containing 100 μg/ml ampicillin of either transformant was diluted 1:100 in 2 l and grown up to 0.7 absorbance units at 600 nm. Induction (5 h) was performed by adding isopropyl-β-D-thiogalactopyranoside up to a final concentration of 0.4 mM. The bacterial cells were collected by centrifugation, re-suspended in buffer A (4 ml/g of wet cells) containing 15% glycerol and 1 mM phenymethanesulfonyl fluoride, and disrupted by pressure using a constant cell disruption system (Constant Systems Ltd., U.K.) at 1.5 kbar. The cellular extracts obtained using this procedure for both D60A and D60E mutants contained the expressed proteins (not shown) and the SsEF-1α mutants were purified using the same procedure already reported for SsEF-1α [27]. To this aim, the post-ribosomal supernatant was treated for 30 min at 70 °C, and most of the *E. coli* denatured proteins were removed by centrifugation. Under these conditions, the sample obtained from the D60A mutant contained a major component soluble protein corresponding to SsEF-1α, whereas that obtained for the D60E one did not; in addition, the absence of the soluble D60ESsEF-1α was observed even when the heat treatment of the cell expressing extract was carried out at 50 °C. Therefore, the supernatant of the D60A mutant cell extract treated at 70 °C was dialyzed against 25 mM MES/KOH buffer (pH 6.0) and applied to a Mono S HR 10/30 column (Pharmacia) equilibrated with the same buffer. D60ASsEF-1α was eluted by a 0–400 mM KCl and among the fractions collected, those containing a single protein band on SDS-PAGE were pooled, concentrated with Aquacide IIa, dialysed against buffer A containing 50% (v/v) glycerol, and stored at −20 °C. Under these conditions, the enzyme was stable for at least 24 months and using the procedure described, roughly 12 mg of purified protein was obtained.

2.3. D60ASsEF-1α assays

The poly(U)-directed poly(Phe) synthesis and isolation of total tRNA and ribosome from *S. solfataricus* were performed as already described [29]. The preparation of [^{3}H]Phe-EctRNA^{Phe}, the formation of the ternary complex between the elongation factor, GppNHp and aa-tRNA, and the protection against the spontaneous deacylation of [^{3}H]Phe-EctRNA^{Phe}, were carried out as described [28].

The ability of D60ASsEF-1α to form a binary complex with [^{3}H]GDP and the determination of the apparent dissociation rate constant of the D60ASsEF-1α·GDP complex was tested by the nitrocellulose filtration method as reported [30]. The number of [^{3}H]GDP binding sites and the apparent equilibrium dissociation constant (K'_d) of the binary complex D60ASsEF-1α·[^{3}H]GDP were determined by a Scatchard plot in presence of 0.5 μM D60ASsEF-1α and 0–25 μM [^{3}H]GDP (specific activity of 1198 cpm/pmol); K'_d for GTP was obtained by competitive-binding experiments in the presence of 5 μM [^{3}H]GDP (specific activity of 2040 cpm/pmol) at different GTP concentrations (50–400 μM).

The intrinsic NaCl-dependent GTPase activity (GTPase^{Na}) was measured in the presence of 3.6 M NaCl as reported [31]. The reaction mixture contained 0.5–1.0 μM purified elongation factor and 50 μM [γ-^{32}P]GTP (specific activity 120–250 cpm/pmol) in 200 μM of buffer B. The reaction was followed kinetically at 60 °C, and the amount of $^{32}P_i$ released was determined on appropriate aliquots. The k_{cat} of GTPase^{Na}, the K_m for [γ-^{32}P]GTP, and the inhibition constants were determined by Lineweaver–Burk plots as reported [31].

2.4. Heat stability of D60ASsEF-1α

Heat inactivation of D60ASsEF-1α was evaluated by measuring the first order kinetic constant of the inactivation process at