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ORIGINAL PAPER

Molecular and functional properties of the psychrophilic elongation factor G from the Antarctic Eubacterium *Pseudoalteromonas haloplanktis* TAC 125

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Abstract The molecular and functional properties of the elongation factor (EF) G from the psychrophilic Antarctic eubacterium *Pseudoalteromonas haloplanktis* (Ph) were studied. PhEF-G catalyzed protein synthesis in vitro that was inhibited by fusidic acid, an antibiotic specifically acting on EF-G. The EF interacted with GDP only in the presence of *P. haloplanktis* ribosome and fusidic acid with an affinity similar to that displayed by *Escherichia coli* EF-G. The psychrophilic translocase elicited a ribosome-dependent GTPase that was competitively inhibited by GDP, the slowly hydrolyzable GTP analog GppNHp, and the protein synthesis inhibitor ppGDP. The temperature dependence of the activity of PhEF-G reached its maximum at least 26°C beyond the growth temperature of *P. haloplanktis* (4–20°C). The heat inactivation profile of the ribosome-dependent GTPase of PhEF-G gave a temperature for half inactivation (46°C), significantly lower than that for half denaturation measured by either UV- (57°C) or fluorescence-melting (62°C). This finding was attributed to a different effect of the temperature on the catalytic domain with respect to that elicited on the other domains constituting the EF, thus confirming the differential molecular flexibility present in psychrophilic enzymes. A molecular model, based on the 3D coordinates of a thermophilic EF-G, showed differences only in connecting loops.

Keywords Elongation factor G · Psychrophilic · *Pseudoalteromonas haloplanktis* · GTPase · Heat stability

Abbreviations

EF	Elongation factor
Ph	*Pseudoalteromonas haloplanktis*
Ec	*Escherichia coli*
Tt	*Thermus thermophilus*
rPhEF-G	Heterologously expressed PhEF-G

Introduction

Extremophiles are organisms living under extreme environmental conditions such as low or high pH (acidophiles and alkaliphiles, respectively), high salt concentration (halophiles), high hydrostatic pressure (barophiles) and high (thermophiles and hyperthermophiles) or low (psychrophiles) temperatures (Herbert and Sharp 1992). Adaptation of proteins to extreme temperatures and the resulting effects on stability and catalytic properties of these mac-

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romolecules have been described in several reports (Zuber 1988; Jaenicke and Zavodszky 1990; D'Amico et al. 2006; Siddiqui and Cavicchioli 2006; Feller et al. 1996). The data obtained indicated that the temperature optimum of enzyme activity represents a compromise between their thermal inactivation and the rate of the catalyzed reaction. Therefore, activity and stability are strictly correlated; indeed, thermophilic enzymes are more exceptionally thermostable and acquire a functional catalytic efficiency at high temperatures (Zuber 1988). More recently, the attention of several investigators has been devoted to cold-adapted enzymes isolated from psychrophilic organisms. These enzymes, although still active at temperatures far beyond their optimum growth condition, are characterized by high catalytic efficiency at temperatures (4–15°C) at which mesophilic or thermophilic enzymes display very low activity (D'Amico et al. 2006; Siddiqui and Cavicchioli 2006; Feller et al. 1996; Feller and Gerday 1997). In addition, they possess the ability to undergo conformational changes that favor the interaction with the substrate(s) thus compensating the slow catalytic rates expected at low temperatures. On the other hand, such flexibility is also related to a thermal instability, a common property of cold-adapted proteins (D'Amico et al. 2006; Siddiqui and Cavicchioli 2006; Feller et al. 1996; Feller and Gerday 1997; Hoyoux et al. 2004). Therefore, psychrophilic enzymes constitute an important class of proteins in which the structure–function relationships can be investigated, also comparing their properties with those of homologous mesophilic and thermophilic counterparts.

The elongation factor G (EF-G in bacteria; EF-2 in eukarya and archaea) is an appropriate molecule for such an investigation, as it is a ubiquitous enzyme and its functional properties and structural features have been extensively studied in a wide variety of mesophilic and thermophilic organisms (Lucas-Lenard 1971; Miller and Weissbach 1977; Kaziro 1978; Stark et al. 2000) and in a psychrophilic archaeon (Thomas and Cavicchioli 2000). EF-G/EF-2 belongs to the GTP-binding protein family (Dever et al. 1987) whose function is related to the alternative binding of GDP or GTP, switching the enzyme from an inactive to an active form, respectively. It is involved in the elongation cycle of protein synthesis catalyzing the translocation of the peptidyl-tRNA from the A site to the P site of the ribosome, thus rendering the A site available for a new elongation cycle (Miller and Weissbach 1977; Kaziro 1978; Stark et al. 2000). EF-G/EF-2 is a multifunctional protein as, in the fulfillment of its biological function, it interacts with nucleotides (Lucas-Lenard 1971; Klink 1985), the ribosome-mRNA complex (Kaziro 1978; Stark et al. 2000, Klink 1985; Baca et al: 1976; Peske et al. 2004); furthermore, it displays a ribosome-dependent GTPase activity (Parmeggiani and Sander 1981; Raimo et al. 1995) and harbors the catalytic site for GTP hydrolysis (De Vendittis et al. 1986; Masullo et al. 1989; Raimo et al. 1996). Finally, antibiotics belonging to the fusidane family, specifically interacting with EF-G and EF-2, have also been identified (Bodley et al. 1969; Willie et al. 1975; De Vendittis et al. 2002). Therefore, an investigation related to function–structure relationships could take advantage of the finding that the biochemical properties of EF-G/EF-2 can be used individually as tools to evaluate the effects produced by the living environment on its functions. Moreover, due to its ubiquity, the amino acid sequence of EF-G/EF-2 has also been used as a tool for phylogenetic relationship establishments in the universal tree of living organisms (Creti et al. 1994; Bocchetta et al. 2000). The characterization of EF-G isolated from a cold-adapted microorganism will enhance our knowledge on the molecular basis of protein stability and on the structure-function relationship in proteins. We have already thoroughly investigated the molecular, physical and biochemical properties of EF-2 isolated from the hyperthermophilic archaeon *Sulfolobus solfataricus* (optimum temperature for growth 87°C) (Raimo et al. 1992, 1995, 1996; De Vendittis et al. 1999, 2002). In this work we describe the biochemical characterization of a heterologously expressed psychrophilic EF-G from *Pseudoalteromonas haloplanktis* TAC 125, an Antarctic eubacterium growing in the temperature interval 4–20°C (Birolo et al. 2000). These results extended the studies on the elongation cycle in *P. haloplanktis* in which the properties of the other two EFs EF-Tu and EF-Ts have already been reported (Masullo et al. 2000; Raimo et al. 2005).

Materials and methods

Materials

Restriction and modifying enzymes, and chemicals were as already reported (Arcari et al. 1999). Nucleotides and fusidic acid (sodium salt) were purchased from Sigma. [γ-^{32}P]GTP (5,000 Ci/mmol), [γ-^{32}P]ATP (10,000 Ci/mmol) and [^{3}H]GDP (12.4 Ci/mmol) were obtained from Amersham. The Ni-NTA Agarose was from Qiagen. *P. haloplanktis* genomic DNA was isolated as reported (Masullo et al. 2000). [^{3}H]Phe-EctRNA^{Phe} and PhEF-Tu were prepared as already reported (Raimo et al. 2005).

Plasmid construction and expression of recombinant PhEF-G

Pseudoalteromonas haloplanktis genomic DNA was used as a template for the PCR amplification of a DNA

fragment containing the gene coding for PhEF-G, taking advantage of the fact that its coding sequence is inserted in a region of the genome containing also the genes coding for the ribosomal protein S7 (*rpS7*) and EF-Tu (AJ249258). The forward primer (EFG1) ATGGC*i*G AA(G)GC*i*AAC(T)AAA(G)GC*i*TT was designed from the amino acid sequence of a highly conserved region of *rpS7* that in *Escherichia coli* corresponded to the amino acid sequence A_{145}EANKAF, whereas the reverse primer (EFG2) GATACCACGTTCGCGTC corresponded to the region of the sequenced PhEF-Tu gene encoding the amino acid fragment E_{56}RERGI (Masullo et al. 2000). The PCR product (2.3 kbp) was cloned into the pGEM T-easy plasmid and sequenced. The recombinant plasmid was then used as a template for the PCR amplification of a 2.1 kbp fragment using the direct AGAGGATCA T(A)TGGCACGTAACAA (EFG3) and the reverse CA AGCTAAAAA(C)(T)(C)(G)A(G)TACAGCGCG (EFG4) primers. Nucleotides in parentheses indicate mismatches to introduce an *Nde*I restriction site at the level of the initial methionine in the direct primer, a *Xho*I restriction site in the reverse primer at the level of the stop codon of the PhEF-G gene; *i* stays for inosine. After digestion with *Nde*I and *Xho*I the PCR fragment was cloned into the *Nde*I-*Xho*I sites of pET22 expression vector and then used to transform *E. coli* BL21(DE3). Transformed cells were grown up to 0.6 OD_{600} and induced with 100 mg/ml IPTG (Inalco). After centrifugation at 3,000*g* for 15 min, cells were re-suspended in 20 mM Tris–HCl buffer, pH 7.8, 10 mM $MgCl_2$, 7 mM β-mercatpoethanol (buffer A), containing 10% glycerol, and disrupted by pressure using a constant cell disruption system (Constant Systems Ltd, UK) at 1.5 kbar. The cell homogenate was then centrifuged at 100,000*g* for 2 h, and the post-ribosomal supernatant (S-100) was used as starting material for the purification of recombinant PhEF-G (rPhEF-G).

Purification of ribosome from *P. haloplanktis*

The purification of ribosome was achieved by adapting a centrifugation procedure already reported for ribosome from *E. coli* (Sander et al. 1975; De Vendittis et al. 1986). Cells of *P. haloplanktis* were grown at 4°C as already reported (Masullo et al. 2000) to the exponential phase of growth, which is reached after 2–3 days of incubation. Cells obtained from a 10-l culture (35–40 g of wet weight) were collected by centrifugation and mechanically disrupted as reported above. The cell homogenate was then centrifuged at 30,000*g* in order to remove the cellular debris and the supernatant was then ultracentrifuged at 100,000*g*. The pellet of this centrifugation was used for the purification of ribosome through a NH_4Cl-washing procedure. With this aim, the ribosomal pellet was dissolved by gentle agitation at 4°C with buffer A containing 500 mM NH_4Cl (RW-buffer) and centrifuged at 30,000*g* for 1.5 h. The pellet was discarded and the supernatant was ultracentrifuged at 100,000*g* for 2 h. The pellet was then dissolved as reported above, layered on a 18% sucrose cushion in RW-buffer and ultracentrifuged for 3.5 h at 100,000*g*. The pellet obtained from the last centrifugation was dissolved in RW-buffer in which the NH_4Cl concentration was reduced to 50 mM, dialyzed against the same buffer containing 60% glycerol and stored at –20°C. In this sample, 1 A_{260} unit was taken to represent 25 pmol of ribosome. The purity of ribosome was assessed by their very low intrinsic GTPase activity (see below).

Poly(Phe) synthesis assay

Poly(U)-directed poly(Phe) synthesis was essentially assayed as reported (Bhargava et al. 2004). The reaction mixture contained: 20 mM Tris–HCl buffer, pH 7.8, 5 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, 440 μM GTP, 1.25 mM phospho*enol*pyruvate, 1.8 U/ml pyruvate kinase, 0.1 mg/ml poly(U), 0.52 μM *P. haloplanktis* ribosome, 0.32 μM [^{3}H]Phe-EctRNA^{Phe} (specific activity 2,100 cpm/pmol), 0.5 μM PhEF-Tu, in the absence or in the presence of increasing rPhEF-G concentration. The poly(Phe) synthesized was assayed as hot trichloroacetic acid insoluble material on 30 μl of the above-mentioned reaction mixture.

GTPase assay

The GTPase activity was measured kinetically as $^{32}P_i$ released from [γ-^{32}P]GTP by the phosphomolybdate method (Sander et al. 1975). Unless otherwise indicated, the reaction mixture contained 0.1–0.2 μM rPhEF-G, 0.5 μM ribosome and 75 μM [γ-^{32}P]GTP (specific radioactivity 250–500 cpm/pmol). At selected time intervals depending on the temperature, aliquots were withdrawn and the reaction was stopped by the addition of an equal volume of ice-cold 1 M $HClO_4$ containing 1 mM KH_2PO_4. Samples were first incubated for 5 min at 0°C and then centrifuged for 10 min at 4°C in order to remove the precipitated ribosomal pellet. An appropriate aliquot of the supernatant was mixed with three vols of 20 mM sodium molybdate and four vols of isopropyl acetate. After a vigorous stirring and a fast spin-down centrifugation (few seconds), an aliquot of the organic phase was counted for radioactivity using a Tri-Carb 1,500 liquid scintillation spectrometer (Packard).

The kinetic parameters of the GTPase reaction were derived from Lineweaver-Burk plots obtained measuring the kinetics of GTP hydrolysis at 30°C at different [γ-^{32}P]GTP concentration (1–75 μM, specific radioactivity 16,500–220 cpm/pmol). Inhibition studies were carried out

by measuring the kinetic parameters of the ribosome-dependent GTPase catalyzed by rPhEF-G in the presence of different inhibitors. K_i values were calculated as reported (Masullo et al. 1994).

GDP binding assay

The GDP-binding ability of rPhEF-G was checked in the presence of fusidic acid by the nitrocellulose filtration method (Baca et al. 1976). Unless otherwise indicated, the reaction mixture contained 0.2–0.5 μM rPhEF-G, 0.5–1.0 μM ribosome, 1 mM fusidic acid and 5–10 μM [^{3}H]GDP (specific radioactivity 500–700 cpm/pmol) in 100 μl of 10 mM Tris–HCl buffer, pH 7.8, 10 mM magnesium acetate, 10 mM NH_4Cl, 5 mM β-mercaptoethanol (buffer B). After an incubation of 15 min at 30°C to reach the equilibrium, the mixture was chilled on ice and two aliquots of 45 μl were filtered through nitrocellulose filters pre-equilibrated with the above-mentioned buffer in which the concentration of fusidic acid was 100 μM. Filters were immediately washed twice with 1 ml of the equilibration buffer, dried and counted for radioactivity. Equilibrium dissociation constant was obtained from a Scatchard plot derived from experiments carried out at different [^{3}H]GDP concentration.

Thermal stability of rPhEF-G

The thermal stability of rPhEF-G was evaluated by heat inactivation profiles and spectroscopic melting curves. In the heat inactivation experiments, a 0.2 mg/ml protein solution was exposed for 10 min at different temperatures (30–60°C) and then immediately chilled on ice for at least 30 min. The residual ribosome-dependent GTPase activity was determined as reported above on aliquots of the treated samples using a final rPhEF-G concentration of 0.2 μM. UV-melting curves of rPhEF-G were obtained in the temperature interval of 20–70°C using a computer-assisted Cary 1E spectrophotometer equipped with an electronic temperature controller. The increase in temperature was set to 0.2°C/min and the difference in the absorbance at 286 and 274 nm was recorded at every degree of increase, normalized between 0 and 100%, and plotted versus the temperature. Fluorescence-melting curves were obtained by setting the increase in the temperature at 0.2°C/min and measuring the fluorescence signal at every degree centigrade increase. The excitation and emission wavelengths were 295 and 342 nm, respectively, and the excitation and emission slits were set to 5 and 10 nm, respectively. The values of fluorescence intensity were corrected for temperature quenching, normalized between 0 and 100% and plotted versus the temperature. In both spectroscopic techniques the concentration of rPhEF-G was 0.2 mg/ml.

Other methods

The isolation of total RNA from *P. haloplanktis* and Northern blotting analysis were carried out as reported (Masullo et al. 2000). The molecular weight of rPhEF-G was determined under native conditions by Gel-filtration on Superdex^{TM} 75 10/300 GL (GE Healthcare); the M_r under denaturing conditions was determined by SDS-PAGE run on 10% polyacrylammide gels, which were stained with Coomassie Brilliant Blue R-250. Western blotting analysis was carried out using His-probe^{TM} antibodies (Santa Cruz) for the specific immuno-detection of His-tagged expressed proteins. The concentration of protein solutions was determined by the method of Bradford (Bradford 1976) using bovine serum albumin as the standard. Multiple alignment of EF-G sequences have been performed using the software CLUSTALW at the ExPASy Proteomics server (www.expasy.ch); the three-dimensional modeling of PhEF-G was carried out using the Swiss-Model service (Peitsch 1996; Guex and Peitsch 1997) and the crystal structure coordinates of *Thermus thermophilus* EF-G (PDB code 1FNM) as template. The 3D-model of PhEF-G was visualized using the *iMol* software (www.pirx.com/iMol).

Results

The PhEF-G encoding gene

In a previous work, a partial coding sequence corresponding to the gene encoding PhEF-G was located upstream the PhEF-Tu gene (*tufA*) (Masullo et al. 2000). Southern blot analysis of *P. haloplanktis* genomic DNA using the radiolabeled 2.1 kbp PCR fragment described above, indicated the presence of only one copy of the PhEF-G gene (not shown); therefore, to clone and sequence the entire PhEF-G gene, a PCR reaction was performed using as template genomic PhDNA, and as primers the oligonucleotides EFG1 and EFG2. The PCR product of about 2.3 kbp was cloned into the pGEM T-easy vector (see Supplementary Figure 1a) and sequenced. The 5′-flanking region of the gene coding for PhEF-G (Supplementary Figure 1b) was 27-nucleotides-long and contained a potential ribosomal binding site (Shine and Dalgarno 1974; Barrick et al. 1994), whereas the 3′-flanking region was 62-nucleotides-long and contained a twofold symmetry segment positioned at 2129–2140, as numbered from the PhEF-G starting codon. Furthermore, a possible binding site for RNA polymerase at positions 2078–2085, and 2108–2113 was also present (Pribnow 1975). As in the case of PhEF-Tu (Masullo et al. 2000) and PhEF-Ts (Raimo et al. 2005) genes, also in the PhEF-G gene synonymous codons most frequently used

were those ending with A and T. To estimate the size of the transcript containing the PhEF-G gene, total RNA from *P. haloplanktis* was analyzed by Northern blotting using the ^{32}P labeled 2.1 kbp PCR product reported in Sect. ''Materials and methods'' as a molecular probe. Two major transcripts, corresponding to RNA molecules of about 3.4 and 1.3 kb, and one less abundant transcript of about 7.5 kb, were detected (Supplementary Figure 2). Both 7.5 and 3.4-kb RNAs, were significantly longer than the size of the PhEF-G gene, whereas the 1.3-kb transcript was shorter and probably corresponded to a RNA molecule coding for PhEF-Tu. In fact, as reported above, the molecular probe used contained part of the PhEF-Tu encoding gene. A dendrogram derived from a multiple sequence alignment of several EF-G sequences (more than 100 sequences, not shown) indicated that the highest degree of amino acid sequence identity was found with EF-G from *Idiomarina loihiensis* (Q5QWB4), a microorganism belonging to the *Alteromonadales* order of the *Gammaproteobacteria* class. The amino acid identity between rPhEF-G and *T. thermophilus* EF-G (TtEF-G), the unique EF-G whose 3D structure has been determined (Al-Karadaghi et al. 1996), is 60%.

Expression and purification of recombinant PhEF-G

To assess the best conditions for the expression of soluble recombinant PhEF-G, the growth of the *E. coli* strain transformed with the plasmid reported in the Sect. ''Methods'', was carried out at 25°C instead of at 37°C. In fact, using the higher growth temperature, although the expression level was significantly higher, rPhEF-G accumulated in the inclusion bodies, from which it could be isolated as an inactive enzyme using denaturing agents such as urea or guanidine. Vice versa, using a growth temperature of 25°C, even though the expression level was reduced, rPhEF-G remained soluble and active (see below). The expression temperature of 25°C was used by others to express human mitochondrial EF-G (Bhargava et al. 2004). Therefore, after the induction with IPTG for 20 h at 25°C the cell extract was obtained and the expressed rPhEF-G was purified by Ni^{2+}-agarose affinity chromatography, whose profile is reported in Supplementary Figure 3a. The analysis on SDS-PAGE (Supplementary Figure 3b) indicated that both at 20 and 50 mM imidazole the elution of a protein band corresponding to the calculated molecular mass of PhEF-G (around 78 kDa) was achieved. Fractions eluted at 20 or 50 mM imidazole containing a single protein band were pooled separately (pool 1 and 2, respectively), dialyzed against buffer A containing 50% (v/v) glycerol and stored at –20°C for further analysis.

The identification of rPhEF-G in the samples eluted from the Ni^{2+}-agarose was achieved by western blotting using commercially available antibodies that specifically identify His-tagged proteins on SDS-gel, and by the determination of the ribosome-dependent GTPase activity. Only the sample eluted at 50 mM imidazole (pool 2) contained a protein reacting with the above-mentioned antibodies (not shown). Furthermore, the measurement of the ribosome-dependent GTPase activity of these samples confirmed the results obtained by western blotting. In fact, the results reported in Fig. 1 indicate that both protein samples exhibited a very low intrinsic GTPase activity, a property exhibited also by ribosome purified from *P. haloplanktis*. Upon the addition of ribosomes to the reaction mixtures containing the protein samples obtained from the above-mentioned chromatography, a significantly increased hydrolytic activity could be measured only when the pool 2 and ribosome were present simultaneously. These results clearly indicate that the protein sample eluted at 20 mM imidazole does not correspond to rPhEF-G; therefore, it was discarded and the sample eluted at 50 mM imidazole was used to characterize the molecular and functional properties of rPhEF-G.

Molecular and functional properties of rPhEF-G

Purified rPhEF-G exhibited an M_r around 78,000 in both native conditions as determined by gel-filtration on

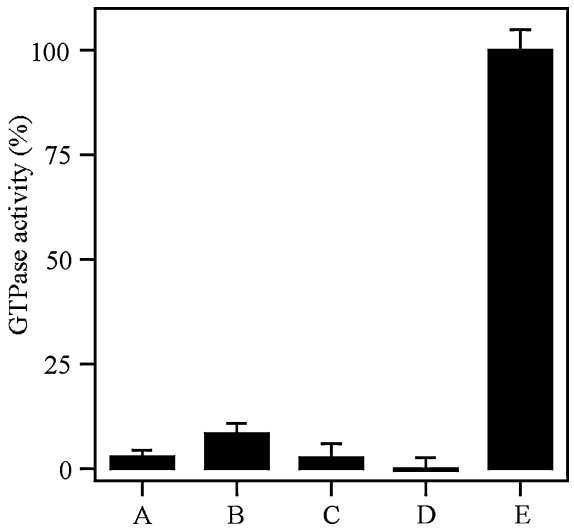

Fig. 1 Ribosome-dependent GTPase of the pools eluted from the affinity chromatography. A 250 µl reaction mixture prepared in buffer A contained 50 µM [γ-^{32}P]GTP (spec. act. 553 cpm/pmol), 0.5 µM ribosome alone (**a**), 18 µg total protein of pool 1 either in the absence (**b**) or in the presence of 0.5 µM ribosome (**c**) or 18 µg total protein of pool 2 either in the absence (**d**) or in the presence of 0.5 µM ribosome (**e**). The reaction was followed kinetically at 15°C and the $^{32}P_i$ released was determined on 50 µl aliquots as reported in Materials and methods. The results were reported as the percentage of the activity obtained in the case **e** (46 pmol [γ-^{32}P]GTP hydrolyzed per minute)

Superdex 75 and denaturing conditions as measured by SDS-PAGE, thus indicating that it functions as a monomeric protein (not shown). The functionality of the EF was assessed by its ability to sustain poly(U)-directed poly(Phe) synthesis, to elicit a ribosome-dependent GTPase activity and to bind GDP only in the presence of fusidic acid, an antibiotic blocking protein synthesis acting specifically on eubacterial EF-G (Bodley et al. 1969; Willie et al.1975).

A reconstituted cell-free *P. haloplanktis* system lacking PhEF-Tu was unable to sustain poly(U)-directed poly(Phe) synthesis even at high concentrations of rPhEF-G. The presence of both EFs promoted phenylalanine polymerization at a level depending on the concentration of rPhEF-G, which remained linear up to a ratio of 1–5 between rPhEF-G and PhEF-Tu (not shown). To better characterize this biochemical property of rPhEF-G, we have measured the effect of temperature on the ability of rPhEF-G to sustain poly(Phe) synthesis. As reported in Fig. 2a, the activity of the psychrophilic EF reached its maximum at 35°C. The analysis in the raising part of the curve according to the Arrhenius equation gave a value of 62.4 kJ/mol for the activation energy. The addition of fusidic acid in the reaction mixture significantly reduced the amount of poly(Phe) synthesized (Fig. 2b) and the inhibition depended on the concentration of the antibiotic. The concentration of fusidic acid leading to 50% inhibition of the activity can be calculated as 3.4 μM.

Regarding the triphosphatase activity, in a first approach we have checked the specificity for the nucleotide triphosphate using either [γ-^{32}P]GTP (Fig. 3a) or [γ-^{32}P]ATP (Fig. 3b) as substrate. Either ribosome from *P. haloplanktis* or rPhEF-G alone possesses a very low intrinsic hydrolytic activity using both substrates; the simultaneous presence of ribosome and rPhEF-G induced a significant increase of the hydrolytic activity only when [γ-^{32}P]GTP was used as substrate (Fig. 3a). Under these conditions, the maximum extent of stimulation was observed at around 1.0 μM ribosome (Fig. 3c).

The ability of rPhEF-G to bind GDP was evaluated by both the inhibition of the ribosome-dependent GTPase activity and more directly by its ability to form a complex with [^{3}H]GDP, fusidic acid and ribosome. To study the inhibition of the ribosome-dependent GTPase of rPhEF-G, kinetic measurements of the GTP breakdown were carried out either in the absence or in the presence of different guanosine nucleotides, known to inhibit the GTPase of EF-G (Arai et al. 1977; Parmeggiani and Sander 1981). The results summarized in Table 1 indicate that both GDP and guanosine tetraphosphate (ppGDP) were strong competitive inhibitors of the ribosome-dependent GTPase of rPhEF-G, whereas the inhibitory power of GppNHp, a not-hydrolyzable GTP analog, is much lower. In fact, the k_{cat} remained substantially unchanged, but the K_m values increased. The highest inhibitory effect was exerted by GDP followed by ppGDP and GppNHp, in that order.

The interaction between rPhEF-G and GDP was also revealed by fusidic acid-dependent nucleotide binding, observed in the presence of *P. haloplanktis* ribosome. The results reported in Fig. 4a indicate that in the absence of ribosome the ability of rPhEF-G to form a complex with [^{3}H]GDP was negligible either in the absence or in the presence of fusidic acid. The addition of ribosome from *P. haloplanktis* significantly increased the amount of the quaternary complex formed. However, the complete

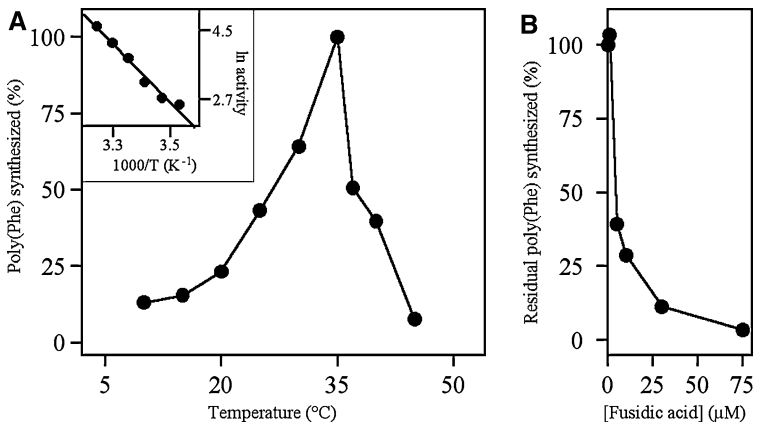

Fig. 2 Poly(U)-directed poly(Phe) synthesis promoted by rPhEF-G. **a** Effect of temperature on the protein synthesis activity catalyzed by rPhEF-G. The reaction mixture was prepared as reported in Materials and methods at the indicated temperature. The results were reported as percentage of the maximum observed at 35°C (11 mmol [^{3}H]Phe incorporated per mol of PhEF-G per minute). *Inset*: the data in the 10–35°C interval were treated according to the Arrhenius equation. **b** Effect of fusidic acid on the poly(Phe) synthesis catalyzed by rPhEF-G. The reaction mixture contained 0.5 μM PhEF-Tu, 0.08 μM rPhEF-G and the indicated concentration of fusidic acid. The amount of [^{3}H]Phe polymerized at 30°C was determined as reported in Materials and methods and reported as percentage of that measured in the absence of the antibiotic (7 mmol [^{3}H]Phe incorporated per mol of PhEF-G per minute)

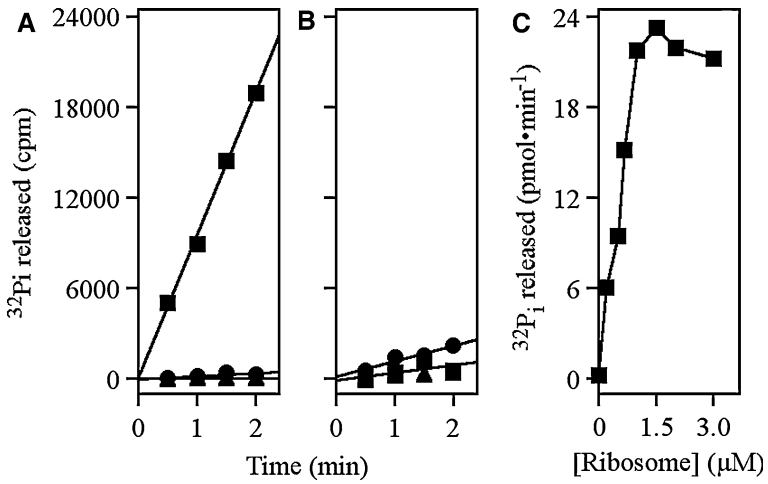

Fig. 3 Ribosome-dependent hydrolytic activity catalyzed by rPhEF-G. **a** and **b** Substrate specificity. The reaction mixture, prepared as reported in Materials and methods in buffer A, contained 0.5 μM ribosome (*dark filled triangle*), 0.25 μM rPhEF-G (*dark filled circle*) or a combination of both (*dark filled square*) in the presence of 50 μM [γ-^{32}P]GTP (spec. act. 486 cpm/pmol (**a**) or [γ-^{32}P]ATP (spec. act. 450 cpm/pmol (**b**). The reaction was carried out at 30°C and at the times indicated the $^{32}P_i$ released was determined on 50 μl aliquots. **c** Effect of ribosome concentration on the GTPase activity of rPhEF-G. The reaction mixture contained 0.25 μM rPhEF-G, 50 μM [γ-^{32}P]GTP (spec. act. 296 cpm/pmol) and the indicated concentration of ribosome in 200 μl of buffer A. At selected time intervals, chosen depending on the concentration of ribosome, the $^{32}P_i$ released was determined on 40 μl aliquots as reported in Materials and methods

Table 1 Kinetic and inhibition parameters of the ribosome-dependent GTPase of rPhEF-G

Nucleotide	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1}μM^{-1})	K_i (μM)
None	15.8	7.3	2.2	–
GDP	18.3	75.2	0.2	2.7
GppNHp	15.0	9.3	1.6	91.5
ppGDP	18.0	37.1	0.5	4.9

The reaction mixture contained 0.2 μM rPhEF-G, 1.0 μM ribosome in the absence or in the presence of 25 μM of the indicated nucleotides. See Sect. ''Materials and methods''

titration of rPhEF-G was not reached, as the maximum amount of complex formation never exceeded 25% of the total protein (Fig. 4b). Similar results were obtained even when the concentration of both the EF and ribosome were different (not shown). However, under the experimental conditions reported in Fig. 4b, the affinity for the nucleotide can be estimated from the slope of the linear graph giving a dissociation constant of the rPhEF-G–[^{3}H]GDP–ribosome–fusidic acid complex of 0.6 μM, a value similar to that measured for the analogous complex formed by EcEF-G (Baca et al. 1976).

Effect of temperature on the activity and the stability of rPhEF-G

The temperature-dependence of the activity of rPhEF-G was evaluated by measuring the ribosome-dependent GTPase of this factor as a function of the temperature. As shown in Fig. 5a, the maximum activity was measured at 45°C, afterwards inactivation occurs. The analysis according to the Arrhenius equation in the raising part of the curve (5–45°C) gave energy of activation of the EF-G-dependent hydrolytic activity of 55.3 kJ/mol, a value very close to that determined by the protein synthesis assay. In addition, the linearity of the plot (Fig. 5b) should rule out the possibility of temperature-induced conformational changes occurring on rPhEF-G. It is worth mentioning that in the temperature interval in which the Arrhenius analysis was carried out, no inactivation of the ability of ribosome to stimulate the rPhEF-G GTPase was observed (not shown).

The thermostability of rPhEF-G was studied by both heat inactivation and spectroscopic-melting curves. The heat inactivation profile reported in Fig. 6a indicates that the ribosome-dependent GTPase of rPhEF-G is half inactivated at 45.9°C, after a ten min exposure. By contrast, the thermal denaturation of rPhEF-G followed by either UV- (Fig. 6b) or fluorescence-melting (Fig. 6c), gave higher half denaturation temperatures of 57.3 and 61.6°C, respectively.

Discussion

In this work, we report the heterologous expression of the gene coding for EF-G from the Antarctic psychrophilic eubacterium *P. haloplanktis*, together with the molecular and functional characterizations of the purified protein. The structural organization of the genome region containing the

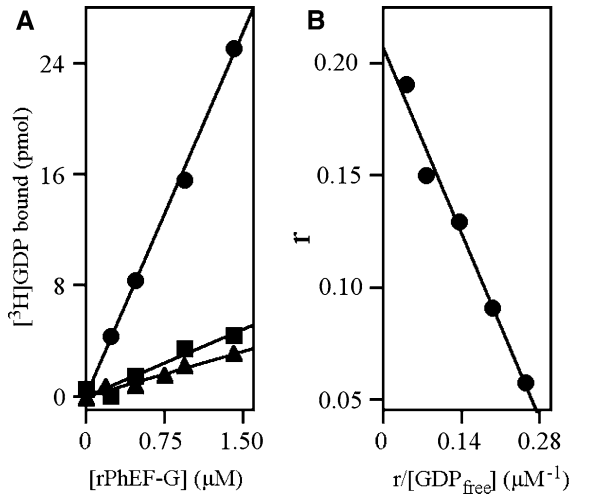

Fig. 4 Binding of [^{3}H]GDP to rPhEF-G. **a** 100 μl reaction mixture prepared in buffer B contained the indicated amount of rPhEF-G and 10 μM [^{3}H]GDP (spec. act. 578 cpm/pmol) in the absence (*dark filled triangle*) or in the presence of 1 mM fusidic acid alone (*dark filled square*) or in combination with 0.5 μM ribosome (*dark filled circle*). The amount of [^{3}H]GDP bound was determined as reported in Materials and methods. **b** 100 μl reaction mixture prepared in buffer B contained 0.47 μM rPhEF-G, 0.5 μM ribosome, 1 mM fusidic acid and 0.13–4.72 μM [^{3}H]GDP (spec. act. 3,706 cpm/pmol). Blanks run in the presence of ribosome and fusidic acid were subtracted and the data were analyzed according to the Scatchard equation. See Materials and methods for other details

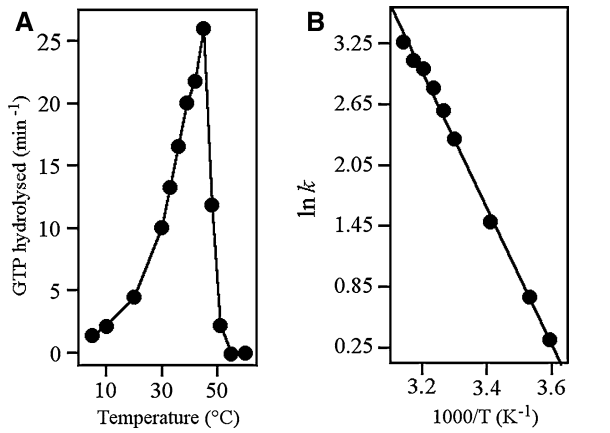

Fig. 5 Thermophilicity of the ribosome-dependent GTPase catalyzed by rPhEF-G. **a** The kinetics of the GTP breakdown was measured at the indicated temperature in 250 μl reaction mixture containing 0.2 μM rPhEF-G, 0.8 μM ribosome and 60 μM [γ-^{32}P]GTP (spec. act. 180 cpm/pmol). At selected time intervals depending on the temperature, the amount of $^{32}P_i$ released was determined on 50 μl aliquots. Blanks run in the absence of rPhEF-G were subtracted. Data were reported as mol of [γ-^{32}P]GTP hydrolyzed per mol of PhEF-G per minute. **b** The data in the 5–45°C interval were treated according to the Arrhenius equation

gene coding for EF-G in this microorganism is similar to the structure of the *E. coli str* operon (Post and Nomura 1980). In fact, also in *P. haloplanktis* the EF-G encoding gene (*fusA*) was preceded by that coding for the ribosomal protein S7 (*rbpS7*) and followed by that for EF-Tu (*tufA*). The length of the intragenic region between *rbpS7* and *fusA* (27 nucleotides) is one-nucleotide shorter than the corresponding region in *E. coli* and containing also an identical ribosomal-binding site sequence. PhEF-G is confined in a high molecular weight transcription unit which did not include *tufA*, as also suggested by the presence of a putative promoter sequence at the 3′-flanking region of *fusA* (Masullo et al. 2000); this behavior is also supported by the finding that the *tufA* transcript is 1.3-kbp long (Masullo et al. 2000). Therefore, the transcriptional unit containing *fusA* probably includes other genes located in its upstream region. In fact, the size of the 3.4-kb transcript (Supplementary Figure 2), was coherent with the length of a transcriptional unit containing the ribosomal proteins S12 (*rpS12*), S7 (*rpS7*) and PhEFG (*fusA*), in the order. This finding is also confirmed by nucleotide sequence analysis of the *P. haloplanktis* genome in this region that revealed the presence of a putative promoter element and of a palindromic sequence only in the intragenic region upstream *rpS12*. In addition, the less abundant larger transcript observed (7.5 kb) could represent a different bigger transcriptional unit that included the gene coding for a RNA polymerase subunit (*rpoC*) located upstream the *rpS12* gene, reminiscent of an additional putative promoter element found in the 5′-flanking region of this gene.

The nucleotide sequence of *fusA* (accession no. AJ249258) is identical to the corresponding sequence identified in the *P. haloplanktis* genome (accession no. CAI85329) deposited more recently (Medigue et al. 2005), except for one silent base substitution (G instead of T) at the position 663. In the nucleotide sequence coding for PhEF-G the synonymous codons most frequently used are those ending in A or T, being the percentage of each base in this position 29.6, 35.8, 17.4, and 17 for A, T, G, C, respectively. These features are also present in the genes coding for PhEF-Tu (Masullo et al. 2000), PhEF-Ts (Raimo et al. 2005), aspartate aminotransferase (Birolo et al. 2000) and the molecular chaperone GroEL (Tosco et al. 2003). In addition, the percentage of base usage in the third position resembled the A/T content (59%) in the whole genome (Medigue et al. 2005). Therefore, it can be surmised that the base composition could represent one of the mechanisms by which DNA responds to the adaptive pressure exerted by temperature.

The nucleotide sequence of the gene coding for PhEF-G shows 61.5% sequence identity toward a second putative EF-G encoding gene (EF-G2, accession no. CAI87975) identified in the *P. haloplanktis* genome. Although this feature is common in the eubacterial kingdom, the presence of a second putative gene coding for EF-G has been found in the genome of other psychrophilic microorganisms such

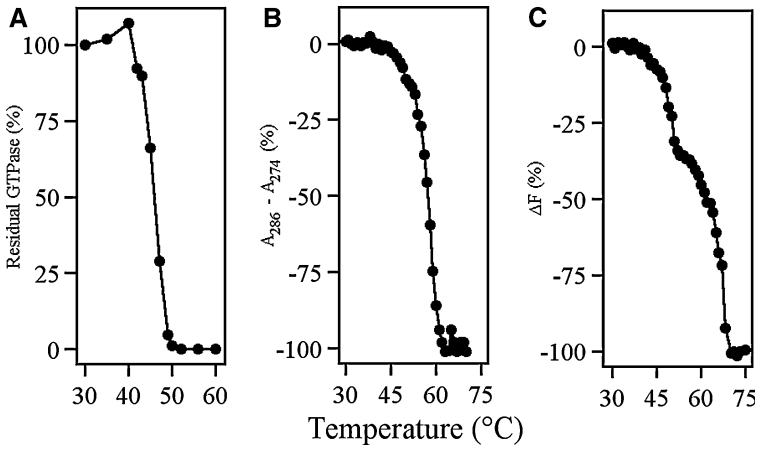

Fig. 6 Thermostability of rPhEF-G. **a** Heat inactivation of the ribosome-dependent GTPase. **b** UV-melting denaturation profile. **c** Fluorescence denaturation profile. Other details are reported in Materials and methods

as *Desulfotalea psychrophila* (NC_006138), *Psychromonas ingrahamii* (NC_008709) and *Colwellia psychrerythraea* (NC_003910).

The translated amino acid sequence of PhEF-G contained the three sequence motifs typical of GTP-binding proteins (Dever et al. 1987), corresponding to the segments A_{17}HVDAGKT, D_{88}TPG and N_{142}KMD; furthermore, the additional two sequence motifs, specifically found in the group of the translational GTPases of GTP-binding proteins, were also identified in the primary structure of rPhEF-G, at the positions G_{59}ITI and G_{267}SA. The primary structure of PhEF-G exhibited high sequence identity with other eubacterial EF-Gs. The high sequence homology between PhEF-G and *T. thermophilus* EF-G (TtEF-G, 55%) allowed the building of a molecular model for the psychrophilic enzyme. The model was generated using the coordinates of the 3D structure of the *T. thermophilus* H573A EF-G mutant as template (Hansson et al. 2005). Although several structures of TtEF-G have been determined, we have chosen this template because of its higher resolution that permitted the solving of domain III structure and the identification of the fusidic acid binding site. The PhEF-G model obtained (Supplementary Figure 3) is almost superimposable to the template except for few differences. In particular, these differences were found in the regions G_{74}MDAQFD, W_{193}NEADQGM, D_{523}ITDDE and E_{644}DAL, all containing a single amino acid insertion, and K_{399}SIIT containing a single amino acid deletion. All these differences were found in connecting loops in the EF-G model.

PhEF-G was identified on the basis of its ability to sustain poly(U)-directed poly(Phe) synthesis in a re-constituted *P. haloplanktis* cell-free system. Furthermore, fusidic acid, an antibiotic blocking eubacterial protein synthesis acting specifically on EF-G, inhibited phenylalanine incorporation.

PhEF-G elicited a very low intrinsic GTPase activity that, as found for other EF-G/EF-2, is strongly enhanced in the presence of ribosome (Sander et al. 1975; Arai et al. 1978; Parmeggiani and Sander 1981; Raimo et al. 1995). The K_m value for GTP of the uncoupled GTPase catalyzed by PhEF-G at 30°C (7.3 μM) was 5.6-fold lower than that displayed by EcEF-G (Parmeggiani and Sander 1981; De Vendittis et al. 1986; Masullo et al. 1989), but closer to that elicited by TtEF-G (Arai et al. 1978). At 15°C the K_m value for GTP was very similar (11 μM) to that measured at 30°C, whereas the k_{cat} was about threefold lower (5.0 min^{-1}). Compared to EcEF-G (Parmeggiani and Sander 1981; Arai et al. 1977), GDP is a more effective inhibitor of the GTPase activity as the K_i for the nucleotide diphosphate was about one order of magnitude lower (2.7 and 36 μM for PhEF-G and EcEF-G, respectively). An opposite behavior was found for the slowly hydrolysable GTP analog, GppNHp, for which the affinity for rPhEF-G was lower (91.5 and 4.3 μM for PhEF-G and EcEF-G, respectively).

Fusidic acid has been shown to increase the stability of the EcEF-G–GDP–Ribosome–FA complex (Baca et al. 1976). Also for PhEF-G a complex with GDP can be formed more efficiently only in the presence of ribosome and FA. Although the titration of PhEF-G by GDP was not complete, the K_d of the complex (0.6 μM) was similar to that reported for EcEF-G (0.38 μM) (Baca et al. 1976); a different behavior was reported for TtEF-G, where the TtEF-G–GDP–Ribosome complex was stable even in the absence of FA (Arai et al. 1978). The interaction between rPhEF-G and fusidic acid was also analyzed from the structural point of view in the 3D-model of the enzyme. Almost all residues involved in the fusidic acid binding site of TtEF-G (Hansson et al. 2005; Johanson et al. 1996; Johanson and Hughes 1994; Martemyanov et al. 2001; Nagaev et al. 2001) were conserved in the psychrophilic enzyme. In fact, only 3 out 30 residues known to induce fusidic acid resistance after mutation/substitution were not conserved in PhEF-G. In particular, L_{113}, C_{114}, and I_{139} in PhEF-G were found in place of F_{108}, D_{109}, and A_{134} in *T. thermophilus*.

The effect of temperature on the properties of PhEF-G was evaluated by measuring both its temperature dependence of the activity and thermostability. Regarding the temperature effect on the activity of the psychrophilic enzyme, it is worth noting that the maximum activity was reached at temperatures (35°C for protein synthesis and 45°C for GTPase) at least 15–25°C higher than that of the growth temperature of *P. haloplanktis*. This result is consistent with that found for the GTPase of PhEF-Tu purified from the same Antarctic eubacterium (Masullo et al. 2000), and could be related to the high flexibility possessed by psychrophilic enzymes (D'Amico et al. 2006; Siddiqui and Cavicchioli 2006; Feller et al. 1996; Feller and Gerday 1997). This behavior is also confirmed by the fact that the average hydrophobicity (Tanford 1962) for amino acid residue in PhEF-G (4.36 kJ/aa) is significantly lower than that of mesophilic EcEF-G (4.56 kJ/aa) and thermophilic TtEF-G (4.84 kJ/aa), respectively. This finding might indicate that in psychrophilic proteins, the space available for amino acid side-chains is larger than that for mesophilic and even more for thermophilic enzymes.

Concerning the thermal stability of PhEF-G, a lower semi-inactivation temperature (46°C) was found, with respect to the half denaturation temperature measured by UV (57°C) or fluorescence (62°C) melting curves. Furthermore, the fluorescence denaturation profile presented a biphasic behavior with an inflection point at around 52°C; this finding could indicate that the temperature affects the structure of the different domains constituting the EF, in a different way. These results were not found for the other two EFs EF-Tu and EF-Ts from *P. haloplanktis* (Masullo et al. 2000; Raimo et al. 2005) in which almost superimposable semi-inactivation and half denaturation temperatures were reported. Therefore, in the case of PhEF-G, the significant difference between the temperature for half inactivation and half denaturation could be reminiscent of the finding that the catalytic domain of the enzyme is more prone to heat inactivation than the other four domains constituting the EF. This behavior is common to that of other psychrophilic enzymes, known as local flexibility (Feller and Gerday 1997; D'Amico et al. 2006; Siddiqui and Cavicchioli 2006).

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