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ORIGINAL PAPER

The magic spot ppGpp influences in vitro the molecular and functional properties of the elongation factor 1α from the archaeon *Sulfolobus solfataricus*

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Abstract Guanosine tetra-phosphate (ppGpp), also known as "magic spot I", is a key molecule in the stringent control of most eubacteria and some eukarya. Here, we show that ppGpp affects the functional and molecular properties of the archaeal elongation factor 1α from *Sulfolobus solfataricus* (SsEF-1α). Indeed, ppGpp inhibited archaeal protein synthesis in vitro, even though the concentration required to get inhibition was higher than that required for the eubacterial and eukaryal systems. Regarding the partial reactions catalysed by SsEF-1α the effect produced by ppGpp on the affinity for aa-tRNA was lower than that measured in the presence of GTP but higher than that for GDP. Magic spot I was also able to bind SsEF-1α with an intermediate affinity in comparison to that displayed by GDP and GTP. Furthermore, ppGpp inhibited the intrinsic GTPase of SsEF-1α with a competitive behaviour. Finally, the binding of ppGpp to SsEF-1α rendered the elongation factor more resistant to heat treatment and the analysis of the molecular model of the complex between SsEF-1α and ppGpp suggests that this stabilisation arises from the charge optimisation on the surface of the protein.

Keywords Elongation factor 1α · *Sulfolobus solfataricus* · Stringent control · ppGpp · Magic spot · Intrinsic GTPase

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Abbreviations

SC	Stringent control
Ss	*Sulfolobus solfataricus*
Ec	*Escherichia coli*
EF	Elongation factor
$GTPase^{Na}$	GTPase activity of SsEF-1α measured in the presence of 3.6 M NaCl
ppGpp	Guanosine tetra-phosphate

Introduction

The accumulation of guanosine tetra- and penta-phosphates (ppGpp and pppGpp, called also magic spot I and magic spot II, respectively, or alarmones) occurs in most eubacteria during stringent control (SC) (Braeken et al. 2006; Magnusson et al. 2005) but not in mutant strains lacking SC, which have been defined relaxed strains (Cashel et al.

1996). Concerning eukaryotes, the presence of alarmones has been demonstrated in plants where these molecules accumulate in chloroplasts upon biotic and abiotic stresses (Takahashi et al. 2004). ppGpp, a compound structurally similar to that of GDP, is characterised by the presence of an additional diphosphate group, bound through a phosphoester bond to the oxygen in position 3′ of ribose. Its production in eubacteria can be also induced by stress conditions or nutritional starvation (Cashel et al. 1996; Battesti and Bouveret 2006). The enzymes involved in magic spot synthesis are the *relA* gene product (p)ppGpp synthetase I and the *spoT* gene product (p)ppGpp synthetase II (Potrykus and Cashel 2008; Atkinson et al. 2011). The latter enzyme possess both synthetic and degrading activity and is responsible for the production of (p)ppGpp regardless SC and amino acid starvation, thus presumably explaining the basal level of (p)ppGpp always present in some bacteria (Potrykus and Cashel 2008; Atkinson et al. 2011). Recently, a growing number of RelA/SpoT homologues, designated RSH, have been identified in plants (Mizusawa et al. 2008) and their role in plant physiology/growth was previously reported (Kasai et al. 2002; van der Biezen et al. 2000). Furthermore, the identification of such proteins in the genome of several sources including some archaea allowed the analysis of phylogenetic relationships among these proteins in the tree of life (Atkinson et al. 2011). Finally, SpoT orthologs have also been recently identified in metazoa, and their involvement in body growth and starvation response in *Drosophila melanogaster* have been demonstrated (Sun et al. 2010).

In bacterial cells, (p)ppGpp plays a role as a negative effector of stable RNA (sRNA) levels and also in several other aspects to SC response such as that of restricting translational errors during amino acid starvation, thus influencing translation accuracy through different proposed mechanisms (Cashel et al. 1996). One of these involves the interaction of magic spots with translation elongation factors EF-Tu and EF-G (Rojas et al. 1984) as well as with the initiation factor IF-2 (Yoshida et al. 1972; Milon et al. 2006; Mitkevich et al. 2010). The inhibition of EF-Tu, EF-G or IF-2 functions might be involved in the control of translation fidelity during protein synthesis. In fact, ppGpp would slow down the accuracy-determining step of the reaction, acting specifically on the GTPase reactions catalysed by both elongation and initiation factors. However, a different mechanism has also been proposed in which the EF-Tu·ppGpp complex, upon its interaction with ribosome, can reduce the rate of peptide bond formation and improves proofreading by increasing the proportion of near-cognate aminoacyl-tRNAs rejected by ribosome (Rojas et al. 1984; Dix and Thompson 1986).

In Archaea very little is known on SC and in those so far studied there is a lack of SC with the exception of the Euryarcheota *Haloferax volcanii* showing a bacterial-like SC (Cimmino et al. 1993), and *Halococcus morrhuae*, showing a SC resembling that of eukarya (Cellini et al. 2004). In Crenarchaeota, the genus *Sulfolobus* appears to contain species that are stringent as in bacteria, but operated in the absence of magic spots; therefore, the absence of (p)ppGpp production has been proposed as an additional criteria to differentiate between Archaea and Bacteria (Cellini et al. 2004). In a previous structural characterisation of the elongation factor 1α, an enzyme isolated from the thermoacidophilic archaebacterium *Sulfolobus solfataricus* (SsEF-1α) endowed with a great thermophilicity and resistance to heat denaturation (Masullo et al. 1991; Granata et al. 2006, 2008), we found a ppGpp molecule bound to the active site of the recombinant protein (Vitagliano et al. 2004). This finding was ascribed to the ppGpp produced in *E. coli* (Cashel et al. 1996) in the heterologous expression of SsEF-1α (Ianniciello et al. 1996). In order to evaluate and to quantify the impact of binding of ppGpp to the properties of translational elongation factors, we here report a functional and molecular characterisation of SsEF-1α in the presence of the magic spot I.

Materials and methods

Chemicals, buffers and enzymes

Labelled compounds and chemicals were as already reported (Masullo et al. 1997). ppGpp was purchased by TriLink BioTechnologies (USA).

The following buffers were used: buffer A: 20 mM Tris·HCl, pH 7.8, 50 mM KCl, 10 mM $MgCl_2$; buffer B: 20 mM Tris·HCl, pH 7.8, 10 mM $MgCl_2$, 1 mM DTT, 3.6 M NaCl.

SsEF-1α was produced and purified as already reported (Ianniciello et al. 1996). SsRibosome, SstRNA, SsEF-2 and SsFRS were purified as reported (Raimo et al. 1992; Lombardo et al. 2002).

SsEF-1α assays

The poly(U)-directed poly(Phe) synthesis was performed at 75 °C as already described (Masullo et al. 2002). The preparation of [^{3}H]Val-EctRNA^{Val}, the formation of the ternary complex between the elongation factor, aa-tRNA and GDP or GTP or ppGpp, and the protection against the spontaneous deacylation of [^{3}H]Val-EctRNA^{Val} were carried out as already reported (Raimo et al. 2000).

The ability of SsEF-1α to exchange [^{3}H]GDP for unlabelled GDP, GTP or ppGpp, the determination of the apparent dissociation rate constant of the SsEF-1α·[^{3}H]GDP complex and the determination of the

equilibrium dissociation constant of the SsEF-1α·ppGpp complex were assessed by the nitrocellulose filtration method as reported (Masullo et al. 1991). Values are indicated as the mean of at least three different experiments with the indication of the standard error.

The GTPase activity was measured in the presence of 3.6 M NaCl ($GTPase^{Na}$) (Masullo et al. 1994). Unless otherwise indicated the reaction mixture contained 0.1–0.3 μM SsEF-1α and 50 μM [γ-^{32}P]GTP (specific activity 150–300 cpm/pmol). The reaction was followed kinetically up to 30 min at 60 °C; the amount of $^{32}P_i$ released was determined on 50 μl aliquots as already reported (Masullo et al. 1994); the catalytic constant of $GTPase^{Na}$, the affinity for [γ-^{32}P]GTP and the inhibition constants for ppGpp of $GTPase^{Na}$ were determined as reported previously (Masullo et al. 1997).

Fluorescence measurements

Heat denaturation of SsEF-1α was studied by fluorescence melting curve realised on a computer assisted Cary Eclipse spectrofluorimeter (Varian) equipped with an electronic temperature controller. The excitation and emission wavelengths were 280 and 311 nm, respectively, and the excitation and emission slits were both set to 10 nm. Blanks run in the absence of the protein were carried out in parallel and subtracted. The fluorescence intensity was corrected for temperature quenching (Underfriend 1969), not observed for the blank, normalised between 0 and 100 % and plotted versus the temperature.

Results

ppGpp inhibited the archaeal protein synthesis in vitro

The ability of ppGpp to affect archaeal poly(U)-directed poly(Phe) synthesis was assessed using a purified cell-free system reconstituted in vitro with the required components isolated from *Sulfolobus solfataricus* (De Vendittis et al. 2002). As reported in Fig. 1a, ppGpp exerted only a weak inhibitory effect on the rate of poly[^{3}H]Phe synthesis catalysed by SsEE-1α; in fact, in the presence of 550 μM ppGpp, a 20 % reduction of the phenylalanine incorporation rate was observed. From the analysis of the effect exerted by different concentrations of ppGpp on poly(Phe) incorporation (Fig. 1b), the concentration leading to 50 % inhibition (IC_{50} = 2.1 mM) was derived from a semi-logarithmic plot, as reported in Fig. 1c.

Moreover, we have also investigated the ability of SsEF-1α to form an heterologous ternary complex with aa-tRNA also in the presence of ppGpp, through the protection exerted by the elongation factor on aa-tRNA against spontaneous deacylation; the results reported in Fig. 2 showed that ppGpp induced an intermediate affinity of SsEF-1α for aa-tRNA when compared with that induced by GDP or GTP.

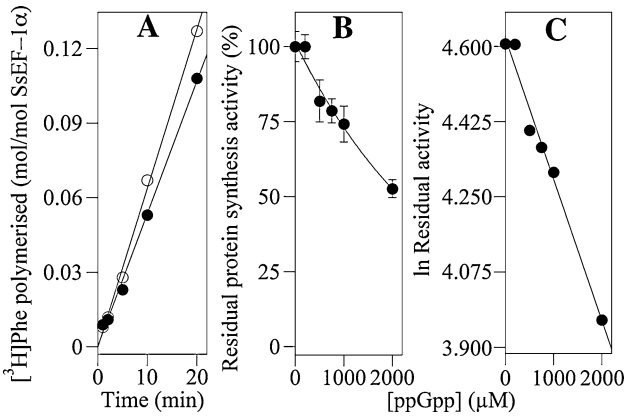

Fig. 1 Effect of ppGpp on the poly(U)-directed poly(Phe) synthesis catalysed by SsEF-1α. **a** 250 μl of the reaction mixture contained 25 mM Tris·HCl pH 7.5, 19 mM magnesium acetate, 10 mM NH_4Cl, 10 mM dithiothreitol, 2.4 mM ATP, 1.6 mM GTP, 0.16 mg/ml poly(U), 3 mM spermine, 0.25 μM Ssribosome, 80 μg/ml SstRNA, 0.1 μM SsEF-2, 2.0 μM [^{3}H]Phe (specific radioactivity 3094 cpm/pmol). The reaction was started by addition of 0.5 μM final concentration of SsEF-1α in the absence (*open circle*) or in presence (*filled circle*) of 550 μM ppGpp and carried out at 75 °C. At the times indicated, 50-μl aliquots were withdrawn, chilled on ice and then analysed for the amount of [^{3}H]Phe incorporated as hot trichloroacetic acid insoluble material. **b** The assay was carried out in triplicate as reported in **a** at the indicated guanosine tetra-phosphate concentration. The standard *error bars* are reported. **c** The data reported in **b** were treated as a semi-logarithmic plot

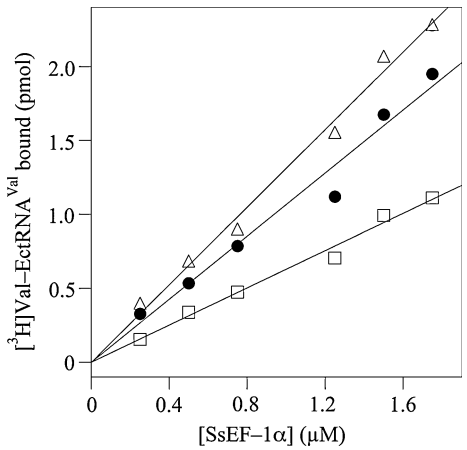

Fig. 2 Formation of the ternary complex between SsEF-1α, [^{3}H]ValEc-tRNA^{Val} and ppGpp or GDP or GTP. The mixture (30 μl) contained 25 mM Tris·HCl, pH 7.8, 10 mM NH_4Cl, 10 mM DTT, 20 mM magnesium acetate and 4.6 pmol of [^{3}H]ValEc-tRNA^{Val} (specific radioactivity 847 cpm/pmol) and was incubated for 1 h at 0 °C to allow ternary complex formation in the presence of the indicated amount of SsEF-1α·GDP (*open square*) or SsEF–1α·ppGpp (*filled circle*) or SsEF-1α·GTP (*unfilled triangle*). The deacylation reaction was carried out for 1 h at 50 °C and the residual [^{3}H]ValEc-tRNA^{Val} was determined as cold trichloroacetic acid insoluble material

ppGpp interacted with SsEF-1α

The affinity of ppGpp for the archaeal elongation factor was measured by competitive binding experiments. SsEF-1α binds [^{3}H]GDP with an affinity in the micromolar range (Ianniciello et al. 1996); as reported in Fig. 3a, the addition of increasing concentration of the tetra-phosphate nucleotide reduced the amount of the SsEF-1α·[^{3}H]GDP complex formation, thus indicating an inhibitory competitive effect. From a semi-logarithmic plot of the data (Fig. 2b), the concentration leading to 50 % inhibition (252 μM) was derived. This value allowed the calculation of the equilibrium dissociation constant for ppGpp (15.7 ± 2.8 μM) which was about one order of magnitude higher than that previously reported for GDP (1.6 μM) and slightly lower than that for GTP (35 μM) (Ianniciello et al. 1996).

Furthermore, SsEF-1α was able to exchange bound [^{3}H]GDP for free guanosine nucleotides (Fig. 4) and the exchange rate for GDP (first order rate constant, $k_{-1} = 0.10 \pm 0.03$ min^{-1}) was faster than that for GTP ($k_{-1} = 0.06 \pm 0.03$ min^{-1}). The intermediate ability of ppGpp to exchange bound [^{3}H]GDP on SsEF-1α ($k_{-1} = 0.08 \pm 0.02$ min^{-1}) confirmed the results observed for the equilibrium dissociation constant.

The interaction between SsEF-1α and ppGpp was also studied analysing the effect of the nucleotide on the intrinsic GTPase^{Na}. The catalytic activity of SsEF-1α was sensitive to the presence of tetra-phosphate; the data reported in Fig. 5 indicated that in the presence of different concentrations of

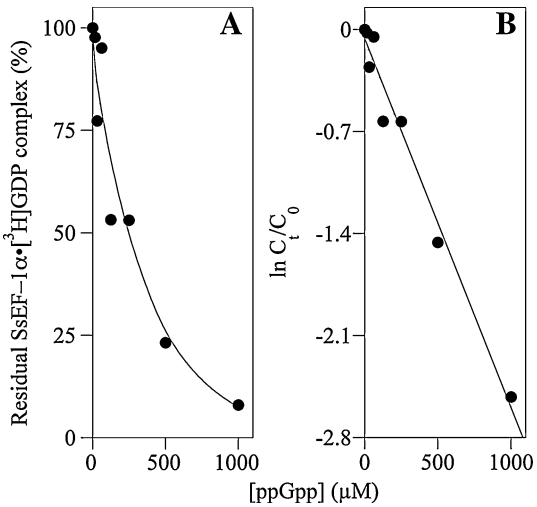

Fig. 3 Competitive binding of [^{3}H]GDP and ppGpp to SsEF-1α. **a** The reaction mixture (50 μl), containing 20 mM Tris·HCl, pH 7.8, 50 mM KCl, 10 mM $MgCl_2$, 25 μM [^{3}H]GDP (specific radioactivity 512 cpm/pmol), 1 μM SsEF-1α and increasing concentration of ppGpp (3–1000 μM), was incubated for 30 min at 60 °C to reach the equilibrium. The amount of the residual SsEF-1α·[^{3}H]GDP complex was determined on 40-μl aliquots by nitrocellulose filtration. **b** Semi–logarithmic plot of data reported in **a**

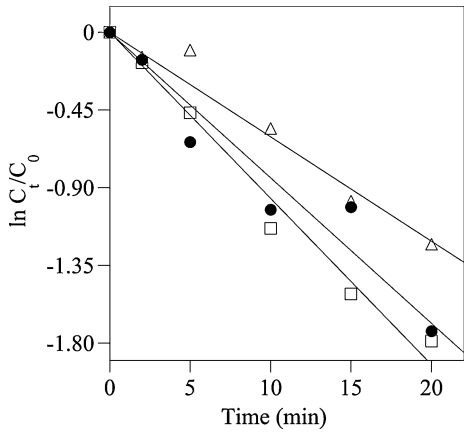

Fig. 4 Guanosine nucleotides exchange rate catalysed by SsEF-1α. The reaction mixture (150 μl) prepared in buffer A contained 1 μM SsEF-1α·[^{3}H]GDP. The nucleotide exchange reaction was started at 60 °C by adding 333 μM GDP (*open square*) or ppGpp (*filled circle*) or GTP (*unfilled triangle*) final concentration. At the times indicated, 30-μl aliquots were filtered on nitrocellulose and the radioactivity retained on the filters was counted. The data were treated according to a first-order kinetic. C_t represents the concentration of SsEF-1α·[^{3}H]GDP at the time t, whereas C_0 is its concentration at the time 0

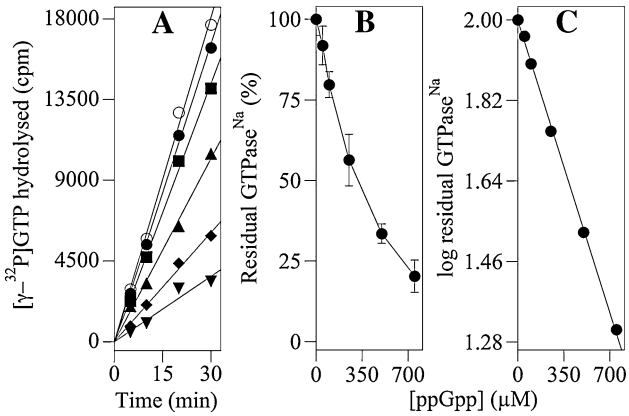

Fig. 5 Effect of ppGpp on the GTPase^{Na} of SsEF-1α. **a** The GTPase activity was assayed at the times indicated, as described in "Materials and methods", in the absence (*open circle*) or in the presence of 50 (*filled circle*), 100 (*filled square*), 250 (*filled upright triangle*), 500 (*filled diamond*) or 750 (*filled inverted triangle*) μM of ppGpp. **b** The rate of GTP hydrolysis derived from three experiments carried out as in **a** were plotted against ppGpp concentration as a percentage of that measured in the absence of the nucleotide tetra-phosphate. Standard *error bars* are indicated. **c** The data reported in **b** were linearised using a semi-logarithmic plot

ppGpp, the rate of GTP breakdown catalysed by SsEF-1α was lower (Fig. 5a, b). The data, linearised using a first-order behaviour equation (Fig. 5c), allowed the evaluation of ppGpp concentration required to get 50 % inhibition of the intrinsic GTPase^{Na} of SsEF-1α (320.5 μM).

In order to get an insight on the inhibition mechanism, the kinetic parameters of the GTPase^{Na} were determined in

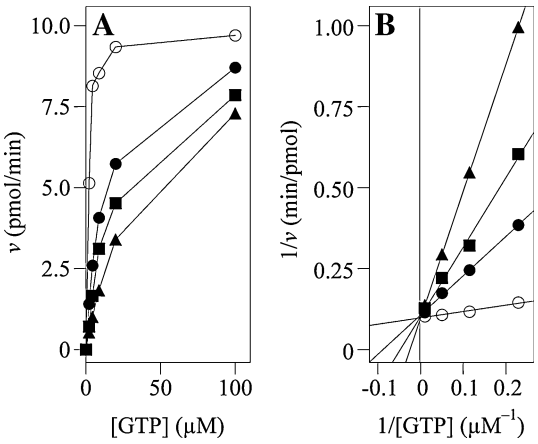

Fig. 6 Affinity of SsEF-1α for ppGpp in GTPase^{Na}. **a** The initial hydrolysis rate (v) was determined at the indicated [γ-^{32}P]GTP concentration in the absence (*open circle*) or in the presence of 75 (*filled circle*), 150 (*filled square*) or 300 (*filled triangle*) μM ppGpp as reported in "Materials and methods". **b** The data reported in **a** were treated with the Lineweaver–Burk equation

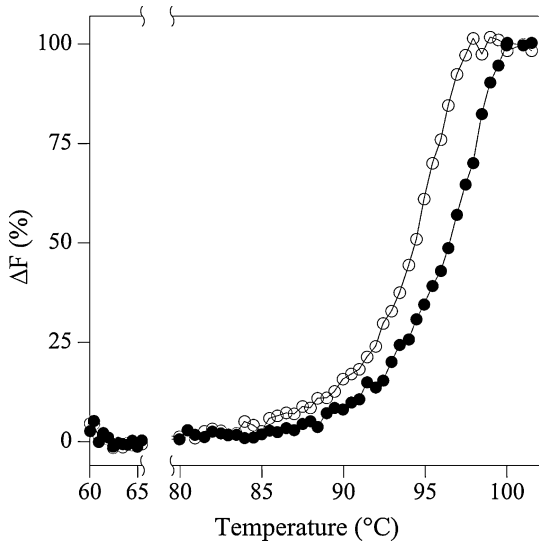

Fig. 7 Effect of ppGpp on the heat denaturation profile of SsEF-1α·GDP. The increase in fluorescence intensity was measured in buffer A at the indicated temperatures using 2 μM SsEF-1α·GDP, in the absence (*open circle*) or in the presence of 20 μM ppGpp (*filled circle*). The temperature increasing rate was set to 0.2 °C/min. In the 65–80 °C interval (omitted in the figure) no fluorescence variation was observed

the presence of different ppGpp concentration (Fig. 6a). The data analysed according to the Lineweaver–Burk equation showed that ppGpp induced an increase of K_m (from 2.0 ± 0.3 μM in the absence to 11.3 ± 1.9, 21.0 ± 2.4 and 40.0 ± 6.1 μM in the presence of 75, 150, and 300 μM ppGpp, respectively) without any variation in the k_{cat} values (from 0.80 ± 0.22 min^{-1} in the absence to a mean value of 0.75 ± 0.26 min^{-1} in the presence of the three ppGpp concentration), thus indicating that the nucleotide tetra-phosphate acted as a competitive inhibitor. It is relevant that the calculated K_i value (15.5 ± 0.3 μM) was almost identical to the value of the equilibrium dissociation constant determined with the competitive binding experiments reported in Fig. 3.

Effect of ppGpp on the thermostability of SsEF-1α

The effect of temperature on the stability of SsEF-1α in the presence of ppGpp was here evaluated by fluorescence-monitored thermal denaturation. As shown in Fig. 7, the denaturation profile of the elongation factor in the presence of ppGpp was shifted towards higher temperatures with a denaturation midpoint (96.4 °C) about 2 °C higher with respect to that observed for the elongation factor bound to GDP. These findings indicated that the extra diphosphate group present in the magic spot I exerted a protective effect against SsEF-1α thermal denaturation.

Discussion

In this work, we have demonstrated that ppGpp, a molecule involved in the stringent control both in eubacteria and plants during amino acid starvation, was able to interact with the archaeal elongation factor 1α from *S. solfataricus* and to influence its molecular and functional properties. A number of experiments here reported unveiled the effects produced by ppGpp on the molecular and functional properties of SsEF-1α. In line with previous crystallographic data showing that ppGpp was found bound to SsEF-1α in the crystalline state (Vitagliano et al. 2004), the ability of magic spot I to interact with the protein was confirmed using different experimental approaches based on the competitive inhibition exerted by ppGpp on the GDP or GTP binding to the elongation factor. The affinity of SsEF-1α towards ppGpp was thoroughly intermediate between that elicited for GDP or GTP.

The interaction of the guanosine tetra-phosphate with the protein affected also both the thermostability and the catalytic activity of SsEF-1α. We have previously shown that the binding of GDP to SsEF-1α increased its thermal stability by approximately 4 °C (Granata et al. 2008). This was not unexpected as this nucleotide makes extensive interaction with protein residues (Vitagliano et al. 2001, 2004). The binding of ppGpp to SsEF-1α produced a further increase of the temperature by 2 °C when compared to GDP. This finding was somehow surprising as the extra diphosphate group present in the ppGpp is solvent exposed and does not make any short range interaction with the protein (Fig. 8). Therefore, the over-stabilisation of protein by ppGpp binding may be explained by considering that SsEF-1α shows, under the experimental conditions used in

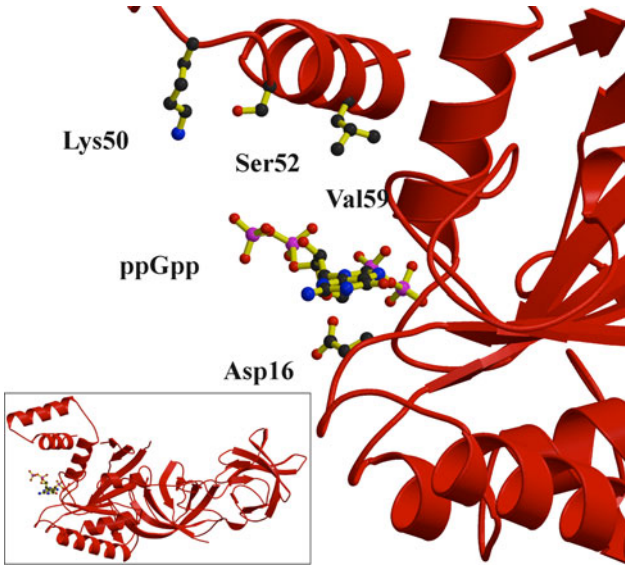

Fig. 8 SsEF-1α nucleotide binding site. The model was generated using the coordinates of SsEF-1α reported in the Worldwide Protein Data Bank (PDB ID: 1SKQ). The position of the terminal phosphate group is indicative as it is not involved in interactions with the protein atoms closer than 8.0 Å and is free to rotate. *Inset* Overall structure of the SsEF-1α·ppGpp complex. The diphosphate group bond to the oxygen in position 3′ of ribose is fully exposed to the solvent

this work but also in the crystallisation experiments, a remarkable overall positive charge being its isoelectric point 9.1 (Masullo et al. 1991). The presence of additional negative charges in ppGpp compared to GDP could lead to an improved charge distribution of the surface of the protein upon magic spot binding.

Significant effects were also observed on the partial reactions catalysed by the elongation factor. Indeed, ppGpp was able to inhibit the intrinsic $GTPase^{Na}$ of SsEF-1α with a dose response behaviour as also recently reported for plant chloroplasts (Nomura et al. 2012). Furthermore, kinetic experiments indicated that the nucleotide tetraphosphate acted as a competitive inhibitor. It is relevant that the inhibition constant obtained (15.5 μM) was almost identical to that determined for the equilibrium dissociation constant of SsEF-1α·ppGpp binary complex (15.7 μM) determined in the absence of NaCl. Therefore, the interaction between the archaeal elongation factor and ppGpp takes place regardless of the presence of the high salt concentration.

The guanosine nucleotide tetra-phosphate was also able to inhibit protein synthesis in vitro, although the concentration required to get half–inhibition ($IC_{50} = 2.1$ mM) was significantly higher than that required for the eubacterial (Legault et al. 1972) and eukaryal systems (Nomura et al. 2012; Manzocchi et al. 1973). Compared to the effects caused by ppGpp on the $GTPase^{Na}$ of SsEF-1α, protein synthesis inhibition required concentration of the magic spot one order of magnitude higher. The explanation of the difference in the concentration of nucleotide to measure an effect is probably due to the finding that the protein synthesis assay was carried out in the presence of several other components, mainly ribosomes, EF-2 and synthetic mRNA, rendering the effect of guanosine tetraphosphate less powerful.

Not only do the overall results reported in this paper confirm previous crystallographic indication about the possibility of ppGpp to interact with SsEF-1α, but also demonstrate that magic spot I interferes with the functional properties of the enzyme.

Apparently, the results here described are in disagreement with the findings that (a) in the genome of this archaeon the genes coding for RelA and SpoT have not been identified, (b) no accumulation of magic spots was found in cell extracts of *S. solfataricus* (Cellini et al. 2004) and (c) a BLAST search using amino acid sequences of RSH family (Atkinson et al. 2011) towards the genome of *S. solfataricus* gives no significative matches (not shown). Although a physiological role of ppGpp in *S. Solfataricus* is yet to be demonstrated, the effects here reported can be ascribed to common structural/functional features of elongation factors isolated from different organisms, independently on the role that magic spots play in different species. Along this line, the finding that ppGpp interacts with an archaeal elongation factor could be exploited to shed light on the molecular mechanisms on which the effect of magic spots on the elongation cycle takes place.

In conclusion, the inhibitory effect exerted by ppGpp on the protein synthesis, here quantified on an archaeal elongation factor is comparable to that recently reported for guanosine nucleotides in other sources (Nomura et al. 2012). Therefore, the binding of ppGpp, characterised by structural requirements similar to those required by GDP, could indicate that the regulation of some cellular processes can be achieved without interfering with the GDP/GTP balance which is important for many other metabolic ways as reported for *Saccharomyces cerevisiae* (Iglesias-Gato et al. 2011).

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