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ARTICLE *in* BIOCHIMIE · SEPTEMBER 2011

Impact Factor: 2.96 · DOI: 10.1016/j.biochi.2011.08.019 · Source: PubMed

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Research paper

The eubacterial protein synthesis inhibitor pulvomycin interacts with archaeal elongation factor 1α from *Sulfolobus solfataricus*

Nicola M. Martucci[a,b,1], Anna Lamberti[b,1], Paolo Arcari[b,c,**], Mariorosario Masullo[b,c,d,*]

[a] Dipartimento di Scienze Farmacobiologiche, Università degli Studi "Magna Graecia" di Catanzaro, Complesso "Ninì Barbieri", I-88021 Roccelletta di Borgia (CZ), Italy
[b] Dipartimento di Biochimica e Biotecnologie Mediche, Università degli Studi di Napoli Federico II, Via S. Pansini 5, I-80131 Napoli, Italy
[c] CEINGE Biotecnologie Avanzate s.c.a r.l., Via Gaetano Salvatore 486, I-80145 Napoli, Italy
[d] Dipartimento di Studi delle Istituzioni e dei Sistemi Territoriali, Università degli Studi di Napoli "Parthenope", Via Medina 40, I-80133 Napoli, Italy

ARTICLE INFO

Article history:
Received 25 May 2011
Accepted 27 August 2011
Available online 10 September 2011

Keywords:
Elongation factor 1α
Sulfolobus solfataricus
Pulvomycin
Protein–antibiotic interaction
Intrinsic GTPase

ABSTRACT

The effect of pulvomycin on the biochemical and fluorescence spectroscopic properties of the archaeal elongation factor 1α from *Sulfolobus solfataricus* (SsEF-1α), the functional analog of eubacterial EF-Tu, was investigated. The antibiotic was able to reduce in vitro the rate of protein synthesis however, the concentration of pulvomycin leading to 50% inhibition (173 μM) was two order of magnitude higher but one order lower than that required in eubacteria and eukarya, respectively. The effect of the antibiotic on the partial reactions catalysed by SsEF-1α indicated that pulvomycin was able to decrease the affinity of the elongation factor toward aa-tRNA only in the presence of GTP, to an extent similar to that measured in the presence of GDP. Moreover, the antibiotic produced an increase of the intrinsic GTPase catalysed by SsEF-1α, but not that of its engineered forms. Finally, pulvomycin induced a variation in fluorescence spectrum of the aromatic region of the elongation factor and its truncated forms. These spectroscopic results suggested that a conformational change of the elongation factor takes place upon interaction with the antibiotic. This finding was confirmed by the protection against chemical denaturation of SsEF-1α, observed in the presence of pulvomycin. However, a stabilising effect of the antibiotic directly on the protein in the complex could takes place.

1. Introduction

Archaeal elongation factor 1α from *Sulfolobus solfataricus* (SsEF-1α) plays a fundamental role in the elongation cycle of protein biosynthesis [1]. SsEF-1α is the functional homologue of the eubacterial elongation factor Tu (EF-Tu), belongs to the class of GTP-binding proteins and possesses an intrinsic GTPase activity revealed in the presence of a molar concentration of NaCl ($GTPase^{Na}$) [2]. The crystal structure of SsEF-1α/EF-Tu showed the presence of three structural distinct domains: a N-terminal domain, containing the nucleotide binding site (G-domain), a middle (M) and a C-terminal (C) domain [3,4]. In its active form complexed with GTP, this EF carries the aminoacyl-tRNA on the ribosome [5]; following codon–anticodon recognition, GTP is hydrolysed, and the resulting inactive form bound to GDP dissociates from the ribosome. The switch from the active to the inactive form is associated to a conformational change of the enzymes. The intervention of the elongation factor Ts/1β, that catalyses the GDP/GTP exchange on the factor, promotes the regeneration of the active form [6,7]. The details of the mechanism of action of *Escherichia coli* EF-Tu (EcEF-Tu) have also been elucidated taking advantage of specific antibiotics acting on the elongation factor. In particular, it has been reported that kirromycin freezes EF-Tu·GDP complex on the mRNA-programmed ribosome by preventing the structural rearrangement of the factor [8]; tetracycline was able to inhibit the binding of aminoacyl-tRNA to the A site of mRNA-programmed ribosome [9]. In addition, pulvomycin and GE2270A interfere with ternary complex formation preventing the interaction between EF-Tu·GTP and aminoacyl-tRNA [8,10]. These antibiotics do not seem to be specific for EcEF-Tu only; in fact, kirromycin was

Abbreviations: 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.

* Corresponding author. Dipartimento di Studi delle Istituzioni e dei Sistemi Territoriali, Università degli Studi di Napoli "Parthenope", Via Medina 40, I-80133 Napoli, Italy. Tel.: +39 081 7463127; fax: +39 081 7463653.
** Corresponding author. Dipartimento di Biochimica e Biotecnologie Mediche, Università degli Studi di Napoli Federico II, Via S. Pansini 5, I-80131 Napoli, Italy. Tel.: +39 081 7463120; fax: +39 081 7463653.
E-mail addresses: arcari@dbbm.unina.it (P. Arcari), mario.masullo@uniparthenope.it (M. Masullo).
[1] These authors equally contributed to this work.

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doi:10.1016/j.biochi.2011.08.019

able to enhance the intrinsic GTPase activity of some SsEF-1α mutants, but not that of the wild-type enzyme [11–13]; GE2270A was found to increase the GDP/GTP exchange rate and to reduce the intrinsic GTPase of the archaeal elongation factor [14]. Furthermore, fusidic acid, another eubacterial antibiotic acting on the elongation factor G, was found to interact with its archaeal functional analogue SsEF-2 [15]. It has to be noted that, in an *in vitro* reconstituted system, none of these antibiotics, except tetracycline, were able to inhibit protein synthesis in *S. solfataricus* [16].

All these findings induced us to investigate the effect of eubacterial antibiotic pulvomycin on the molecular and functional properties of SsEF-1α. The results obtained either by fluorescence spectroscopic analysis or through the effect produced by the antibiotic on the elongation factor showed that pulvomycin was able to interact with the SsEF-1α.

2. Materials and methods

2.1. Chemicals, buffers and enzymes

Labelled compounds and chemicals were as already reported [17]. Pulvomycin powder was a gift from Prof. Andrea Parmeggiani and it was used as 28 mM stock solution in absolute ethanol and stored at −80 °C. The spectral quality of the antibiotic was checked as reported [18] and its concentration was determined using a molar absorbance coefficient of 74 582 M^{-1} cm^{-1} at 320 nm in methanol. 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 and its engineered or chimaeric forms were produced and purified as already reported [17,19,20]. SsRibosome, SstRNA, SsEF-2 and SsFRS were purified as reported [21,22].

2.2. SsEF-1α assays

The preparation of [^{3}H]Val-EctRNAVal, the formation of the ternary complex SsEF-1α·GTP·[^{3}H]Val-EctRNAVal, the protection against spontaneous deacylation of [^{3}H]Val-EctRNAVal and poly(U)-directed poly([^{3}H]Phe) synthesis were carried out as already described [5,14,21].

The ability of SsEF-1α to bind [^{3}H]GDP or to exchange the radiolabelled nucleotide for GDP or GTP was assayed by nitrocellulose filtration as described [17]. Following the titration of 1.0 μM SsEF-1α with 0.4–4 μM [^{3}H]GDP (specific radioactivity 3398 cpm/pmol), the apparent equilibrium dissociation constant (K_d') of the binary complex formed between SsEF-1α and [^{3}H]GDP was determined using Scatchard plots. K_d' for GTP was derived through competitive binding experiments [17] in which 0.5 μM SsEF-1α was incubated in the presence of 10 μM [^{3}H]GDP (specific radioactivity 800 cpm/pmol) and different concentrations (40–300 μM) of GTP.

The intrinsic NaCl-dependent GTPase activity ($GTPase^{Na}$) was measured in the presence of 3.6 M NaCl as reported [2]. The reaction mixture contained 0.5 μM purified elongation factor and 25 μM [γ-^{32}P]GTP (specific activity 400–900 cpm/pmol) in 200 μL of buffer B. The reaction was followed kinetically at 50 °C, and the amount of $^{32}P_i$ released was determined on 40 μl 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 [2].

The effect of pulvomycin on the thermophilicity of the $GTPase^{Na}$ was evaluated by determining the initial velocity of [γ-^{32}P]GTP breakdown in the 45–75 °C interval [7]; the data were then treated with the Arrhenius equation

$$\ln v = \ln A + E_a/R \cdot 1/T$$

in which v is the rate of GTP hydrolysis (s^{-1}) at a given temperature T (K), A is the Arrhenius constant (s^{-1}), E_a is the energy of activation (J mol^{-1}), and R is the gas constant (8.314 J mol^{-1} K^{-1}). By plotting ln v against $1/T$, the E_a can be derived from the slope of the straight-line obtained. The energetic parameters of activation ΔH^*, ΔS^* and ΔG^* were calculated at a given temperature by the equations

$$\Delta H^* = E_a - (R \cdot T) \quad \Delta S^* = R \cdot \ln(h \cdot N_A \cdot A/R \cdot T \cdot e) \quad \Delta G^* = \Delta H^* - T\Delta S^*$$

where h is the Plank constant (6.624×10^{-34} J s), N_A is the Avogadro's number (6.023×10^{23} molecules/mol), and e is the base of the natural logarithm (2.718).

The effect of pulvomycin on the chemical denaturation of SsEF-1α was evaluated by exposing the protein to guanidine hydrochloride (GuHCl, Sigma–Aldrich) as already reported [23], determining the residual $GTPase^{Na}$. To this aim, a stock solution of GuHCl (6.6 M) was prepared and mixed in different amounts with protein solutions to give a constant final value of the protein concentration (10 μM) and a variable concentration of GuHCl (0–6 M). Each sample was incubated overnight at room temperature and GuHCl-induced denaturation was evaluated by assaying the residual GTPase activity bringing each sample to a 0.6 M constant value of GuHCl concentration. Under these conditions, the $GTPase^{Na}$ of SsEF-1α was not affected at all.

2.3. Fluorescence measurements

Fluorescence spectra were recorded at 25 °C on a computer assisted Cary Eclipse spectrofluorimeter (Varian) at a scan rate of 60 nm/min using an excitation wavelength of 280 nm; excitation and emission slits were set to 10 nm. Spectra recorded in the presence of pulvomycin were obtained after further additions of a concentrated solution of the antibiotic and a correction for the dilution was applied. Blanks run in the absence of the protein were subtracted. The analysis of the binding affinity was carried out as already reported [24,25] considering that the quenched fluorescence (Q) at respective maximum is a function of the maximum possible quenching (Q_{max}) at an infinite ligand concentration. In particular, the binding affinity of the protein·pulvomycin complex (K_d) was calculated from the equation

$$Q = (Q_{max} \cdot [\text{Pulvomycin}])/(K_d + [\text{Pulvomycin}])$$

through a double reciprocal plot.

3. Results

3.1. Effect of pulvomycin on the functional properties of SsEF-1α

The interaction between SsEF-1α and pulvomycin was studied analysing the effect produced by the antibiotic on the functional properties of the elongation factor.

In a first approach, the ability of pulvomycin to inhibit protein synthesis in a reconstituted system containing purified macromolecular components from *S. solfataricus* was investigated; the antibiotic, at 200 μM concentration, reduced the poly(U)-directed poly(Phe) synthesis rate of about 4-fold (Fig. 1A). The inhibition data obtained at different pulvomycin concentrations (Fig. 1B), treated through a first-order analysis (Fig. 1C), allowed the extrapolation of the concentration of antibiotic leading to 50% inhibition (173 μM).

The effect of the antibiotic was also studied on the partial reactions catalysed by SsEF-1α. Concerning ternary complex formation between Val-$EctRNA^{Val}$, SsEF-1α and guanosine nucleotides, evaluated through the ability of the elongation factor to

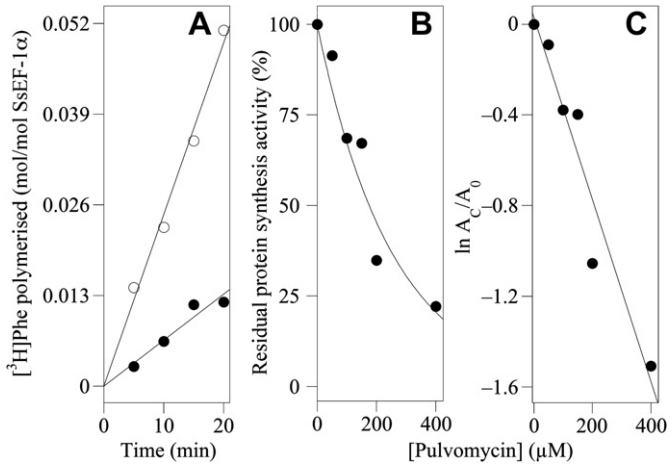

Fig. 1. Effect of pulvomycin on the poly(U)-directed poly(Phe) synthesis at 75 °C. *A* Kinetics of the [^{3}H]Phe incorporation in the absence (open symbol) or in the presence (filled symbol) of 200 μM pulvomycin. The reaction mixture (250 μl) contained 0.5 μM SsEF-1α, 0.25 μM Ssribosome, 80 μg/ml SstRNA, 0.1 μM SsEF-2, 2.0 μM [^{3}H]Phe (specific activity 1514 cpm/pmol) in 25 mM Tris·HCl, pH 7.5 buffer supplemented with 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) and 3 mM spermine. At the times indicated, 50 μl aliquots were withdrawn and analysed for the amount of [^{3}H]Phe incorporated. *B* Effect of different concentrations of pulvomycin. *C* The data reported in *B* were treated as a first-order behaviour. A_0 represent the activity measured in the absence of pulvomycin, whereas A_C is the activity at the concentration *C* of the antibiotic.

protect [^{3}H]Val-EctRNAVal against spontaneous deacylation [5], pulvomycin was able to decrease the affinity of the elongation factor toward aa-tRNA only in the presence of GTP (Fig. 2A), to an extent similar to that measured in the presence of GDP (Fig. 2B).

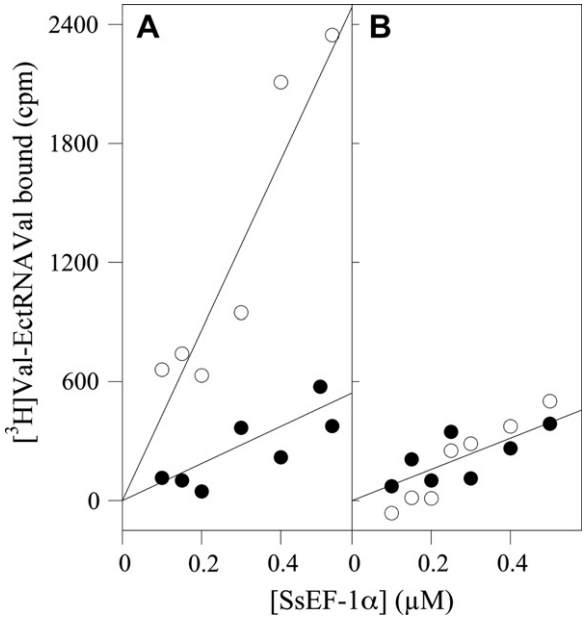

Fig. 2. Effect of pulvomycin on the formation of the ternary complex between SsEF-1α, [^{3}H]ValEc-tRNA^{Val} and GTP or GDP. The reaction mixture (30 μl) prepared in 25 mM Tris·HCl, pH 7.8 buffer, 10 mM NH_4Cl, 10 mM DTT, and 20 mM magnesium acetate, contained 4.6 pmol of [^{3}H]ValEc-tRNA^{Val} (specific activity 1185 cpm/pmol). To allow ternary complex formation the mixture was incubated for 1 h at 0 °C in the presence of the indicated amount of SsEF-1α·GTP (*A*) or SsEF-1α·GDP (*B*), in the absence (open symbols) or in the presence (filled symbols) of 20 μM pulvomycin. The deacylation reaction was then carried out for 1 h at 50 °C and the residual [^{3}H]ValEc-tRNA^{Val} was determined as cold trichloroacetic acid insoluble material.

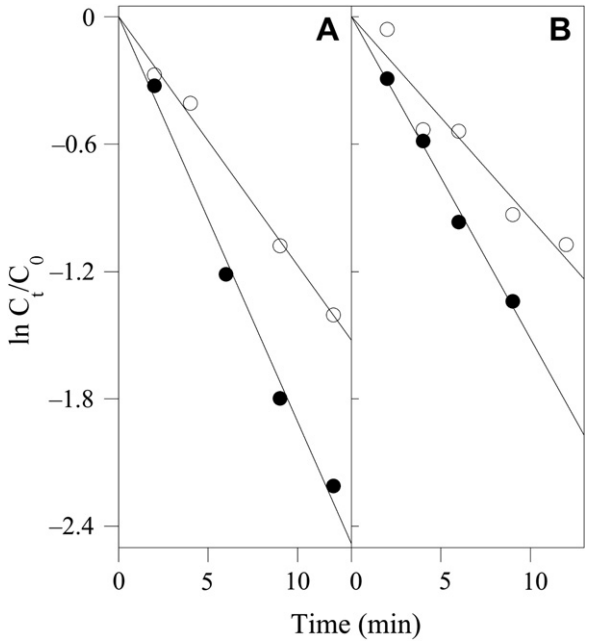

Fig. 3. Effect of pulvomycin on the guanosine nucleotide exchange on the SsEF-1α·[^{3}H]GDP complex. The reaction mixture (250 μl) prepared in buffer A contained 0.5 μM SsEF-1α·[^{3}H]GDP in the absence (empty symbols) or in the presence (filled symbols) of 20 μM pulvomycin. The nucleotide exchange reaction was started at 60 °C by adding 1 mM GDP (A) or GTP (B) final concentration. At the times indicated, the amount of the residual radiolabelled binary complex was determined on 50 μl aliquots by nitrocellulose filtration. The data were treated according to a first-order kinetics.

3.2. Effect of pulvomycin on the interaction between SsEF-1α and guanosine nucleotides

The effect of pulvomycin on the interaction between the elongation factor and guanosine nucleotides was assessed on both the exchange rate and affinity. In presence of pulvomycin, the guanosine nucleotides exchange rate on the archaeal elongation factor was 1.5-fold faster for both GDP (Fig. 3A) and GTP (Fig. 3B). In addition, in the presence of the antibiotic, the guanosine nucleotides apparent equilibrium dissociation constants for GDP and GTP were slightly lower (Table 1) and the effect produced was more evident in the case of GTP. Regarding the slight increased affinity for GDP, the effect of pulvomycin can be ascribed to a higher increase of the association constant with the respect to the dissociation one.

The interaction between pulvomycin and SsEF-1α, was also studied by its effect on the GTPase^{Na}. As reported in Fig. 4A, increasing pulvomycin concentration increased the rate of the intrinsic GTPase catalysed by SsEF-1α, reaching its maximum stimulation effect at 30 μM. This stimulation was not observed for two truncated forms of the archaeal elongation factor lacking the C-terminal (Ss(GM)EF-1α) or the C- and the M-domains (Ss(G)EF-1α) [17], as well as for an engineered elongation factor constituted by

Table 1
Effect of pulvomycin on the affinity of SsEF-1α for guanosine nucleotides at 60 °C.

SsEF-1α	K_d'		k_{-1}	k_{+1}
	GDP (μM)	GTP (μM)	GDP (min^{-1})	GDP (μM^{-1} min^{-1})
− pulvomycin	0.35 ± 0.10	4.7 ± 1.5	0.13 ± 0.02	0.37
+ pulvomycin	0.30 ± 0.11	2.6 ± 2.0	0.19 ± 0.01	0.63

The K_d' and k_{-1} values represent the average of 3–4 different determinations. k_{+1} was calculated as k_{-1}/K_d'.

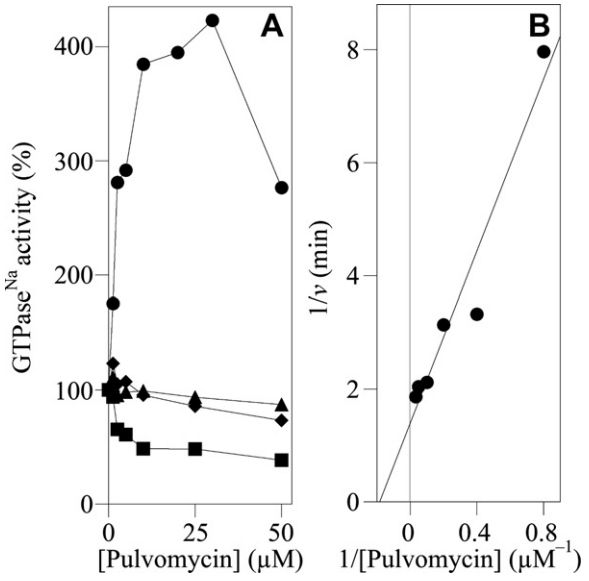

Fig. 4. Effect of pulvomycin on the intrinsic GTPase of SsEF-1α and its engineered forms. *A* The GTPase^{Na} of SsEF-1α (●), Ss(GM)EF-1α (■), Ss(G)EF-1α (▲), and the archaeal/eubacterial chimaeric elongation factor (◆) was determined in the presence of the indicated pulvomycin concentration, as described in Materials and methods. The data were reported as a percentage of the activity measured in the absence of the antibiotic. *B* The data referring to SsEF-1α up to 30 μM pulvomycin were treated by the Lineweaver–Burk equation after the subtraction of the GTPase^{Na} activity of SsEF-1α measured in the absence of pulvomycin (0.166 min^{-1}).

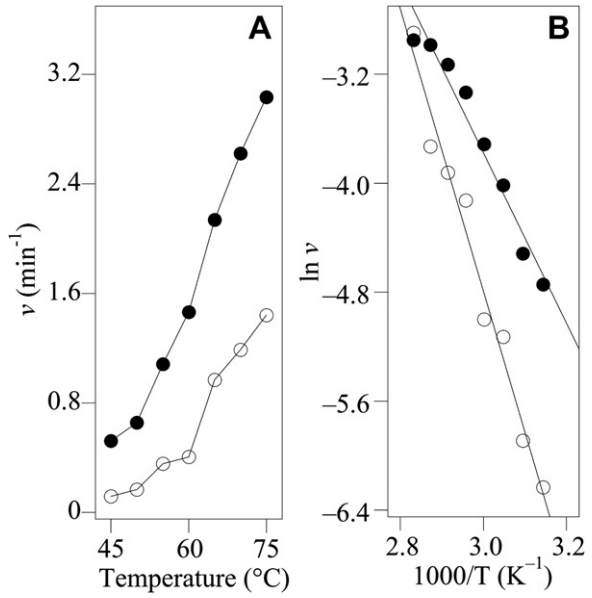

Fig. 5. Effect of pulvomycin on the thermophilicity of the GTPase^{Na} catalysed by SsEF-1α. *A* The rate of GTP breakdown was determined as reported in the Methods section at the indicated temperature, in the absence (empty symbols) or in the presence (filled symbols) of 20 μM pulvomycin. At each temperature, the times used for the determination were selected in order to give a linear relationship between the time and the amount of [γ-^{32}P]GTP hydrolysed. *B* Arrhenius analysis of the data reported in *A*.

the G-domain of SsEF-1 and the M- and C- domains of EcEF-Tu [20]. The data referring to SsEF-1α up to a concentration of pulvomycin of 30 μM followed a saturation behaviour and, through a double reciprocal plot (Fig. 4B), the concentration of pulvomycin required for 50% stimulation (5.4 μM) can be derived from the *X*-axis intercept. The effect of the antibiotic on the kinetic parameters of the GTPase^{Na} was also investigated. The data reported in Table 2 indicated that the increased catalytic efficiency, measured in the presence of 20 μM pulvomycin can be ascribed to an increased affinity for the substrate together with an higher hydrolytic rate. Furthermore, the GTPase^{Na} was competitively inhibited by GDP and the slowly hydrolysable GTP analog GppNHp also in the presence of pulvomycin. In particular, the inhibition power, evaluated through the comparison of the inhibition constants (Table 2), was reduced by 4-fold for both nucleotides, thus confirming the observed increased affinity for the guanosine nucleotides in the presence of the antibiotic.

The effect of pulvomycin on the thermophilicity of GTPase^{Na} was also analysed in the 45–75 °C temperature interval and the antibiotic exerted its stimulatory function at all the tested temperatures (Fig. 5A). The analysis of data through the Arrhenius equation, gave a straight-line also in the presence of the antibiotic, but with a different slope with respect to that obtained in its absence (Fig. 5B). From these data the energetic parameters of activation of GTPase^{Na} in absence or in presence of pulvomycin were calculated and reported in Table 3. The antibiotic reduced the energy of activation of the hydrolytic reaction without changing the free energy of activation. However, a differential effect on the other thermodynamic parameters of activation was observed; in particular, pulvomycin produced a favourable variation of enthalpy of activation that is accompanied by an unfavourable variation of the entropy.

3.3. Effect of pulvomycin on chemical denaturation of SsEF-1α

The effect of antibiotic on the resistance of SsEF-1α against guanidine hydrochloride denaturation was investigated by measuring its residual GTPase activity in the presence of guanidine hydrochloride at increasing concentrations, in the presence or in the absence of 20 μM pulvomycin (Fig. 6). The concentration of denaturant agent to get 50% inactivation of the GTPase^{Na} was 3.0 M in the absence and 3.6 M in the presence of the antibiotic. These results indicated that pulvomycin exerted a protective action against chemical denaturation of SsEF-1α.

3.4. Effects of pulvomycin on fluorescence spectra

The effect of pulvomycin on the molecular properties was also studied by fluorescence spectroscopy in the aromatic region of the fluorescence spectrum (λ_{exc} 280 nm) of SsEF-1α and its truncated forms and compared to that observed for EcEF-Tu. As shown in Fig. 7, pulvomycin exerted for all proteins investigated, a strong

Table 2
Effect of pulvomycin on the kinetic parameters of the GTPase^{Na} catalysed by SsEF-1α at 50 °C.

	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} μM^{-1})	K_i (GDP) (μM)	K_i (GppNHp) (μM)
− pulvomycin	4.36 ± 1.8	0.18 ± 0.04	0.04	0.12 ± 0.04	0.23 ± 0.08
+ pulvomycin	2.61 ± 1.0	0.47 ± 0.14	0.18	0.47 ± 0.10	0.93 ± 0.15

The K_m and k_{cat} values represent the average of 3–4 different determinations.

Table 3
Effect of pulvomycin on the energetic parameters of activation of GTPase^{Na} of SsEF-1α.

	E_a (kJ mol^{-1})	ΔH^a (kJ mol^{-1})	ΔS^a (J mol K^{-1})	ΔG^a (kJ mol)
− pulvomycin	86	83	−36	95
+ pulvomycin	53	50	−127	92

[a] Calculated at 60 °C.

fluorescence quenching at λ_{max} which was accompanied by the appearance of a new peak at 360 nm; this behaviour can be explained by a combination of both a static and a dynamic quenching [26], as well as by an inner filter effect [27] due to the absorption of pulvomycin at the emission region of spectrum (λ_{max} 320 nm); therefore, these findings impaired the correction for the inner filter effect [26]. Moreover, the presence of a new peak at a higher wavelength could indicate a conformational modification induced by antibiotic involving specific aromatic residues, mainly tryptophans [26]. However, a differential effect on quenching and new peak appearance was exerted by pulvomycin on the different proteins analysed. In particular, a prevalence on the quenching effect was observed in the case of SsEF-1α (Fig. 7A), whereas a higher effect on the new peak appearance was observed in the case of the truncated forms of the elongation factor lacking the C-terminal (Fig. 7B) or both the C- and M- domains (Fig. 7C), but also for EcEF-Tu (Fig. 7D). These results, although to a lesser extent, were also observed using an excitation wavelength of 295 nm (not shown).

The analysis of the quenching effect allowed an evaluation of the binding process [24,25] through the variation of the quenched fluorescence (Q) against pulvomycin concentration (Fig. 8A). In fact, the linearity of the double reciprocal plot (Fig. 8B), allowed the extrapolation of the binding constant from the abscissa axis intercept. The values obtained indicated that intact SsEF-1α and EcEF-Tu showed a similar affinity for the drug being the K_d 11.6 μM and 13.9 μM, respectively. In the case of SsEF-1α the lacking of C-terminal domain lowered the affinity of the elongation factor towards pulvomycin ($K_d = 23.9$ μM), whereas the truncation of both the C- and M-domains induced a less evident effect ($K_d = 16.6$ μM).

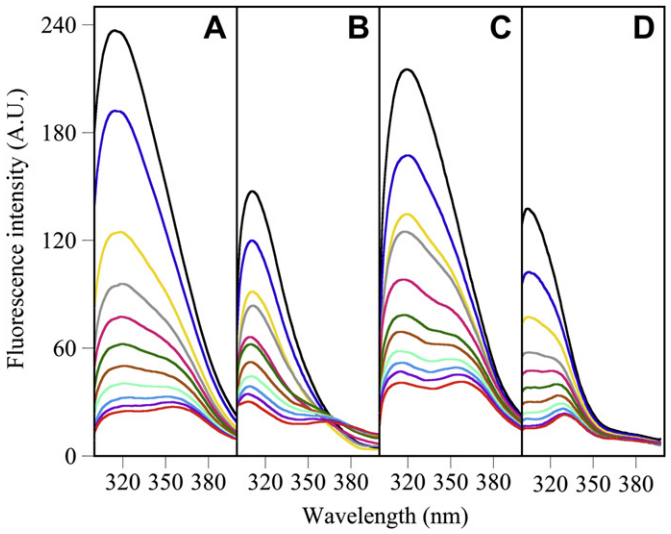

Fig. 7. Effect of pulvomycin on fluorescence spectra of SsEF-1α and its engineered forms. Spectra were recorded in buffer A in the presence of 4 μM SsEF-1α (*A*), Ss(GM)EF-1α (*B*), Ss(G)EF-1α (*C*) or EcEF-Tu (*D*) in the absence (black line) or in the presence of increasing concentration of pulvomycin ranging between 4 (blue) and 38 μM (red) shown by different colors. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

4. Discussion

In this work, several aspects of the action of pulvomycin, on the molecular and functional properties of SsEF-1α were investigated. The results obtained indicated that this eubacterial antibiotic, isolated from *Streptoverticillium netropsis* [18], was able to interact with the archaeal elongation factor. It was previously reported that pulvomycin at 1 mM final concentration was not able to inhibit

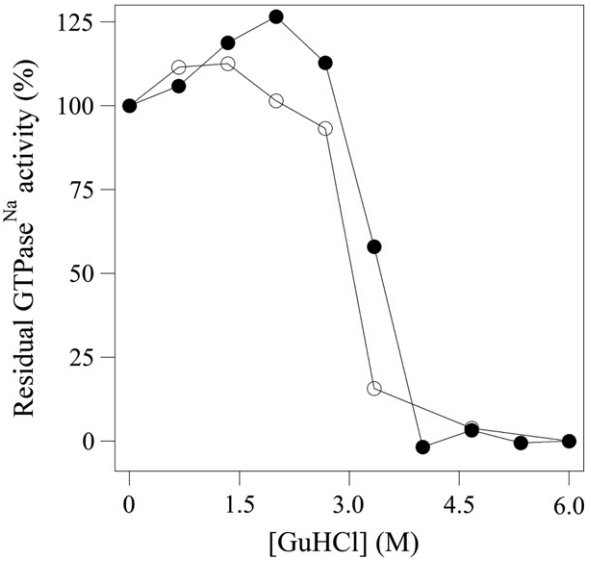

Fig. 6. Effect of pulvomycin on the chemical denaturation of SsEF-1α. The residual GTPase^{Na} was determined, after incubation of the samples at indicated guanidine hydrocloride concentration, as reported in Materials and methods, in the absence (empty symbols) or in the presence (filled symbols) of pulvomycin.

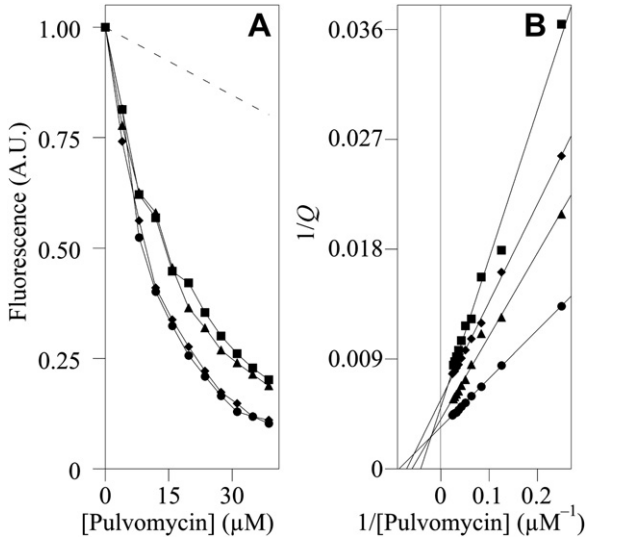

Fig. 8. Quenching analysis of the interaction between pulvomycin and SsEF-1α or its engineered forms. *A* The fluorescence at the respective maximum was plotted against increasing concentration of pulvomycin for SsEF-1α (●, 314 nm), Ss(GM)EF-1α (■, 311 nm), Ss(G)EF-1α (▲, 319 nm), and EcEF-Tu (◆, 310 nm). The dashed line reports the effect on the fluorescence of bovine serum albumin (346 nm), used as a negative control for the interaction. *B* Double reciprocal plot of the data reported in *A*.

protein synthesis in a reconstituted cell-free system from *S. solfataricus* [16]. The availability of purified macromolecular components of *S. solfataricus* required for an *in vitro* protein synthesis assay showed that pulvomycin was able to inhibit poly(U)-directed poly(Phe) synthesis; however, the concentration of antibiotic required to get 50% inhibition (173 μM) was at least two order of magnitude higher than that measurable for both gram-negative and gram-positive eubacterium [28,29], and some methanogenic archaea [30], but at least one order of magnitude lower than that required for *Saccharomyces cerevisiae* [30] or other eukaryotic cells [31]. These findings prompted us an investigation on the effect exerted by pulvomycin on the partial reactions catalysed by SsEF-1α. The results obtained indicated that the antibiotic affected the different functional properties of the elongation factor but at concentrations significantly lower than that required for the inhibition of protein synthesis. This difference can be explained by the finding that the protein synthesis assay was carried out in the presence of several components, mainly ribosomes, EF-2 and synthetic mRNA, rendering the effect of the antibiotic less powerful. However, no effect was exerted by pulvomycin on the intrinsic (Supplementary material Fig. S1) and ribosome-dependent (Supplementary material Fig. S2) GTPase catalysed by SsEF-2. Therefore, a specific effect of pulvomycin on both ribosome and EF-2 can be excluded. On the other hand, this behaviour was also reported for the effects exerted by two other eubacterial antibiotics acting on the partial reactions catalysed by SsEF-1α [14,32]. Regarding the effects on the partial reactions, the presence of the antibiotic reduced the ability of SsEF-1α to form a ternary complex only in the presence of GTP, and the affinity for the aa-tRNA became very similar to that measured using GDP. These findings indicated that, as found for EcEF-Tu, even for an archaeal elongation factor the impairment of the ternary complex formation was the target of this antibiotic action [28].

Regarding the effect of the antibiotic on the interaction with guanosine nucleotides, the results obtained indicated that pulvomycin increased the rate of nucleotide exchange for both GDP and GTP. More pronounced effects were instead observed when the $GTPase^{Na}$ was used as a probe to study the antibiotic interaction. In particular, pulvomycin was able to stimulate the intrinsic NaCl-dependent GTPase catalysed by SsEF-1α, a finding that was already reported for the intrinsic GTPase of EcEF-Tu [33], even though in that case the stimulatory effect was more pronounced. However, the stimulatory effect exerted by pulvomycin cannot be detected for the catalytic activity elicited by the truncated forms of SsEF-1α lacking the C- or the C- and M-domains. These results indicated that the integrity of the elongation factor was required to observe the effect, and that the C-terminal domain was essential for the interaction. The analysis of the energetic parameters of activation could give an explanation of the increased hydrolytic rate of the reaction, measured in the presence of pulvomycin. Indeed, the reduced energy of activation, induced by the antibiotic, was essentially due to a reduced enthalpy of activation, even though an unfavourable entropy factor leads to an unvaried free energy of activation.

The interaction between the archaeal elongation factor and pulvomycin was also demonstrated by the finding that the antibiotic rendered SsEF-1α slightly more resistant to guanidine hydrochloride denaturation. This finding is indicative of a more compact molecular organisation of the elongation factor observed in the presence of pulvomycin. However, a direct stabilising effect of the antibiotic on the protein in the complex cannot be excluded.

Finally, the data obtained from fluorescence spectroscopy, suggested a conformational change induced by the antibiotic upon its interaction with SsEF-1α and confirmed those already reported for EcEF-Tu [8,33]. However, the integrity of the archaeal elongation factor is required to bind the antibiotic with higher affinity. Furthermore, the appearance of a fluorescence band at 360 nm in the presence of pulvomycin for all proteins investigated, is indicative of the exposition to the aqueous solvent of tryptophan residues, even though a resonance energy transfer between close tyrosines and tryptophan(s) residues [26] cannot be excluded. In the primary structure of SsEF-1α two tryptophans and ten tyrosines were present, most of them in the nucleotide binding domain (W209, Y84, Y122, Y161, Y166, Y180, Y210, Y218). Therefore, the finding that the appearance of the 360 nm band was also evident on the truncated forms of the elongation factor, indicated that the main target of the solvent exposition upon antibiotic binding, was the region comprising Trp209 (Fig. 9) to which the resonance energy transfer could occur from tyrosines 161, 166 and 210, surrounding this residue.

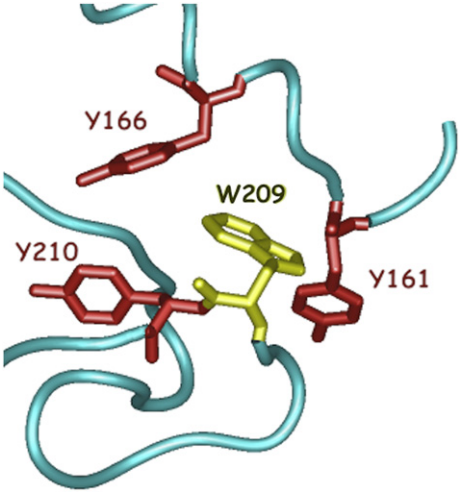

Fig. 9. Close-up of the three dimensional structure of the nucleotide binding domain of SsEF-1α (PDB code 1JNY) surrounding W209. Tyrosines 161, 166 and 210 are indicated in red; tryptophan 209 in yellow. The picture was generated using the software iMol (http://www.pirx.com/iMol). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

5. Conclusions

In conclusion, the data presented strongly indicated a molecular interaction between an archaeal elongation factor 1α and the eubacterial antibiotic pulvomycin. These findings, besides being useful for studying the interaction between pulvomycin and eukaryotic elongation factor 1α, could be used as probe to investigate phylogenetic relationships among living domains.

Acknowledgements

This work was supported by MiUR, PRIN 2007 and 2009 to MM and PRIN 2008 to PA. We are grateful to Prof. Andrea Parmeggiani for critical reading of the manuscript and for providing samples of pulvomycin.

Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.biochi.2011.08.019.

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