Fusidic and Helvolic Acid Inhibition of Elongation Factor 2 from the Archaeon *Sulfolobus solfataricus*†

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ABSTRACT: Fusidic acid (FA) and helvolic acid (HA) belong to a small family of naturally occurring steroidal antibiotics known as fusidanes. FA was studied for its ability to alter the biochemical properties supported by elongation factor 2 isolated from the archaeon *Sulfolobus solfataricus* (*Ss*EF-2). Both poly(Phe) synthesis and ribosome-dependent GTPase (GTPase^r) were progressively impaired by increasing concentrations of FA up to 1 mM, whereas no effect was measured in the intrinsic GTPase of *Ss*EF-2 triggered by ethylene glycol in the presence of barium chloride (GTPase^g). The highest antibiotic concentration caused inhibition of either poly(Phe) synthesis or GTPase^r only slightly above 50%. A greater response of *Ss*EF-2 was observed when HA was used instead of FA. HA caused even a weak impairment of GTPase^g. A mutated form of *Ss*EF-2 carrying the L452R substitution exhibited an increased sensitivity to fusidane inhibition in either poly(Phe) synthesis or GTPase^r. Furthermore, both FA and HA were able to cause impairment of GTPase^g. The antibiotic concentrations leading to 50% inhibition (IC_{50}) indicate that increased fusidane responsiveness due to the use of HA or the L452R amino acid replacement is mutually independent. However, their combined effect decreased the IC_{50} up to 0.1 mM. Despite the difficulties in reaching complete inhibition of the translocation process in *S. solfataricus*, these findings suggest that fusidane sensibility is partially maintained in the archaeon *S. solfataricus*. Therefore, it is likely that *Ss*EF-2 harbors the structural requirements for forming complexes with fusidane antibiotics. This hypothesis is further evidenced by the observed low level of impairment of GTPase^g, a finding suggesting a weak direct interaction between the archaeal factor and fusidanes even in the absence of the ribosome. However, the ribosome remains essential for the sensitivity of *Ss*EF-2 toward fusidane antibiotics.

Elongation factor 2 (EF-2)[1] represents the eukaryal/archaeal counterpart of eubacterial elongation factor G (EF-G), the enzyme that catalyzes translocation during polypeptide elongation. This step of translation is essentially unchanged even in taxonomically distant species, and it is coupled to a GTPase reaction that is required for the release of EF-G and/or EF-2 from the ribosome (for reviews, see refs *1*−*3*). Impairment of translocation is caused by various compounds acting on EF-2 or EF-G. Early observations proved that the naturally ocurring steroidal antibiotic fusidic acid (FA) is the typical inhibitor of EF-G. FA belongs to a small family of antibiotics, known as fusidanes, possessing a similar tetracyclic ring structure with a unique chair−boat−chair conformation and a carboxylic acid-bearing side chain. FA locks the eubacterial EF-G on the ribosome through the formation of a stable ribosome·EF-G·GDP·FA complex (*4*−*6*). Similarly, the eukaryal/archaeal EF-2 is inactivated by an ADP ribosylation reaction catalyzed by diphtheria toxin (*7*−*9*). More recently, the effect of two additional inhibitors of eukaryal EF-2 has been reported. Sordarin inhibits translocation by acting on fungal EF-2 (*10*) with a mechanism different from that observed with FA, and aromatic bisamidine 1 blocks the translocation process supported by EF-2 from rat liver (*11*).

The studies of the mode of action of FA in eubacteria clearly indicate that the specific target of this antibiotic is the eubacterial EF-G (*4*, *5*). However, no direct evidence of the formation of a binary complex between EF-G and FA exists, and only a putative structure of the antibiotic binding site has been derived from the EF-G structure (*12*−*14*). Less information about the mode of action of FA in eukarya exists (*15*, *16*), because of the lower sensitivity. The possibility that even in eukarya the main effect of FA is the impairment of the release of EF-2 from the ribosome cannot be ruled out. However, some evidence has been presented that FA interferes with the phosphorylation state of EF-2 (*17*). Moreover, a possible inhibitory effect of FA on the ADP ribosylation of eukarial EF-2 has been suggested (*18*).

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[1] Abbreviations: EF, elongation factor; *Ss*, *Sulfolobus solfataricus*; L452R *Ss*EF-2, *Ss*EF-2 carrying the L452R substitution; FA, fusidic acid (sodium salt); HA, helvolic acid (sodium salt); PAGE, polyacrylamide gel electrophoresis; GTPase^r, ribosome-dependent GTPase; GTPase^g, intrinsic GTPase triggered by ethylene glycol in the presence of $BaCl_2$; IC_{50}, concentration of antibiotic causing 50% inhibition of activity; PCR, polymerase chain reaction.

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Previous studies on the effect of FA in archaea (*19*) were unsuccessful in finding an inhibitory effect of FA on EF-2, because no inhibition was found in a crude polypeptide elongation system made by a mixture of ribosome and the soluble protein fraction from the hyperthermophilic archaeon *Sulfolobus solfataricus*. Most of the components participating to polypeptide elongation in *S. solfataricus* have now been purified, including EF-2 (*Ss*EF-2) (*20*). The overall percentage of identical amino acids in *Ss*EF-2 and eubacterial EF-G is approximately 30% (*21*). However, among 21 amino acid positions of EF-G related to the FA resistance (*22*), 14 are conserved in *Ss*EF-2. For this reason, we decided to reconsider the effect of FA on the properties of purified *Ss*EF-2 to reveal the possible responsiveness to inhibition by fusidanes even in an archaeal system. These studies could therefore be of value in establishing the minimum structural requirements for the putative antibiotic binding site located on EF-G or EF-2.

This work describes the effects of FA and helvolic acid (HA), another member of the fusidane family, on the biochemical properties of *Ss*EF-2 and its mutated form carrying the L452R substitution. The data that have been obtained indicate that minimum structural requirements for fusidane inhibition are probably conserved in the archaeon *S. solfataricus*.

MATERIALS AND METHODS

Chemicals, Enzymes, and Buffers. All chemicals were analytical grade. Restriction and modifying enzymes were from Amersham or Promega. Labeled compounds were from Amersham, except [γ-^{32}P]GTP which was purchased from NEN. Oligonucleotides were synthesized by Genset (Paris, France). Fusidic acid (FA) and helvolic acid (HA) were purchased from Sigma. Both fusidanes were used as sodium salts from a stock solution prepared by titration of fusidane with sodium hydroxide.

The following buffers were used. Buffer A consisted of 25 mM Tris-HCl (pH 7.5), 19 mM magnesium acetate, 10 mM NH_4Cl, 10 mM dithiothreitol, 1 mM ATP, 0.5 mM GTP, 3 mM spermine, and 0.16 mg/mL poly(U). Buffer B consisted of 50 mM Tris-HCl (pH 7.5), 2 mM magnesium acetate, 50 mM KCl, and 1 mM dithiothreitol. Buffer C consisted of 20 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 0.4 mM NH_4Cl, 10 mM magnesium acetate, 0.05 mg/mL poly(U), 1 mM spermine, and 1 mM ATP. Buffer D consisted of 20 mM Tris-HCl (pH 7.5), 8 mM $BaCl_2$, and 40% (v/v) ethylene glycol.

Preparation of S. solfataricus Macromolecular Components. Total *Ss*tRNA, ribosome, and native *Ss*EF-1α were purified from *S. solfataricus* cells as previously described (*23*). The crude source of aa-tRNA synthetases, constituted by S-100 deprived of *Ss*EF-1α and *Ss*EF-2, was prepared from *S. solfataricus* as previously described (*23*, *24*). Recombinant *Ss*EF-2 was obtained via the expression system previously described (*24*), constituted by the host *Escherichia coli* strain JM109(DE3) (Novagen) transformed with the vector vEF-2 engineered from the plasmid pT7-7 (U.S. Biochemical).

Production of the Mutant SsEF-2 Containing the L452R Amino Acid Replacement. A mutant *Ss*EF-2 was obtained by site-directed mutagenesis of the corresponding gene. The target triplet was T_{1357}TA (for coordinates of the *Ss*EF-2 gene, including the start codon, see ref *21*), and two mismatches allowed its conversion into A_{1357}GA; the corresponding amino acid replacement was L452R (amino acid numbering excludes the initial methionine missing in native *Ss*EF-2; see ref *20*). Using vEF-2 as a template, two different PCR amplifications were carried out on a DNA thermal cycler from Perkin-Elmer by using two couples of oligos; a 944 bp segment was obtained from the direct primer 5′d-A_{1347}TCATTACAA*AG*ATTACGTGAA$_{1368}$-3′ and the reverse primer 5′d-A_{2290}GTCAACTAATGTATCATC$_{2272}$-3′; the other 521 bp segment was obtained from the direct primer 5′d-A_{848}AGCAATGTTAAATGCTGA$_{866}$-3′ and the reverse primer 5′d-T_{1368}TCACGTAAT*CT*TTGTAATGAT$_{1347}$-3′ (the designed mismatches are denoted with italics). The segments were purified on a 1% agarose gel and then mixed at equimolar amounts; after the fragments were melted, reannealing was achieved in the overlapping 22mer region containing the mutated A_{1357}GA triplet of the *Ss*EF-2 gene. The heteroduplex with recessed 3′-ends was extended by *Taq* DNA polymerase and amplified using the direct primer 5′d-A_{848}AGCAATGTTAAATGCTGA$_{866}$-3′ and the reverse primer 5′d-A_{2290}GTCAACTAATGTATCATC$_{2272}$-3′. The resulting 1443 bp segment was shortened to 1217 bp via double digestion with *Kpn*I and *Bst*EII. This segment was cloned in the 3.83 kb *Bst*EII−*Kpn*I fragment prepared from vEF-2. The structure of the new plasmid, called vEF-2(L452R), was identical to that of vEF-2 and contained the designed mutated triplet, as controlled by nucleotide sequencing of the synthetic region, using the T7 sequencing kit from Pharmacia. The procedure followed for the heterologous expression of the new plasmid and for the purification of the mutant L452R *Ss*EF-2 was identical to that described previously (*24*, *25*).

Other Methods. Transformation of bacterial strains, preparation of plasmids, labeling of probes, and other details of DNA recombinant technology were as described previously (*26*). Poly(U)-directed poly(Phe) synthesis supported by *Ss*EF-2 or L452R *Ss*EF-2 was assessed in buffer A as described previously (*23*); GTPase activity in the presence of ribosome (GTPase^r) was measured in buffer C (*27*), whereas the intrinsic GTPase (GTPase^0) was tested in buffer D (*28*). The activities of *Ss*EF-2 and its mutant form were measured either in the presence or in the absence of antibiotics. The activity rates were calculated from the slope of linear kinetics; the values obtained in the absence of antibiotics were compared with those obtained in the presence of increasing concentrations of antibiotics. The antibiotic concentration leading to a 50% reduction of the activity (IC_{50}) was extrapolated from plots of the activity rate versus the logarithm of the antibiotic concentration present in the assay.

Protein purity was evaluated by SDS−PAGE (*29*). Proteins were determined by the method of Bradford (*30*) using bovine serum albumin as the standard.

RESULTS

Inhibition Properties of Fusidic Acid and Helvolic Acid on the Archaeal SsEF-2. Previous studies showed that *Ss*EF-2 is endowed with a great thermophilicity and heat resistance (*20*, *27*, *28*), thus indicating the functional adaptation of this archaeal protein to the optimum growth temperature of *S.*

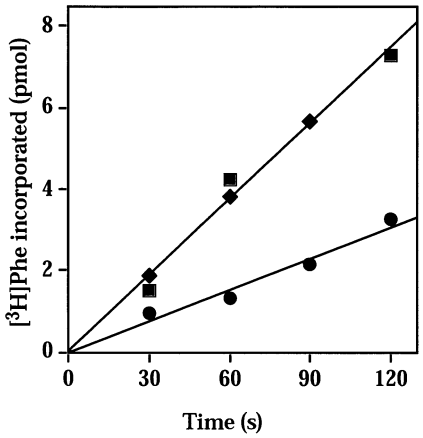

FIGURE 1: Effect of sodium fusidate and sodium chloride on the kinetics of poly(Phe) synthesis supported by *Ss*EF-2. The reaction mixture contained, in a final volume of buffer A of 250 μL, 0.4 μM *S. solfataricus* ribosome, 0.16 mg/mL *Ss*tRNA, 0.015 mg/mL *Ss*Phe-tRNA^{Phe} synthetase, 0.7 μM *Ss*EF-1α, and 0.1 μM *Ss*EF-2 in the absence (■) or in the presence of 1 mM sodium fusidate (●) or 1 mM NaCl (◆). The reaction was carried out at 85 °C and started with the addition of [^{3}H]Phe up to a final concentration of 2.0 μM (specific activity, 438 cpm/pmol). At the indicated times, 50 μL aliquots were withdrawn from the reaction mixture and analyzed for [^{3}H]Phe incorporated as described in Materials and Methods. Blanks run in the absence of *Ss*EF-2 were run in parallel and subtracted.

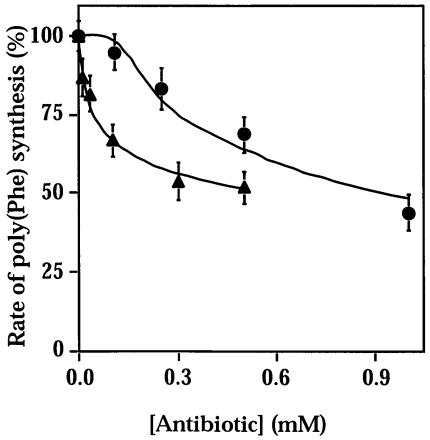

FIGURE 2: Effect of fusidane antibiotics on poly(Phe) synthesis supported by *Ss*EF-2. The rate of poly(Phe) synthesis supported by *Ss*EF-2 in the presence of the indicated concentrations of sodium fusidate (●) or sodium helvolate (▲) was calculated from kinetics performed as indicated in the legend of Figure 1. The results were expressed as percentages of the rate calculated in the absence of antibiotic [0.011 mol of Phe incorporated (mol of *Ss*EF-2)$^{-1}$ s^{-1}].

solfataricus ($T = 87$ °C). Therefore, high-temperature values were used to check the biochemical properties supported by *Ss*EF-2, in an effort to find a possible responsiveness of the archaeal factor to fusidane inhibition.

Among the biochemical properties of *Ss*EF-2, the poly-(U)-directed poly(Phe) synthesis represents the tool used to check the overall functions of the factor in translocation. Figure 1 shows the kinetics of poly(Phe) synthesis supported by *Ss*EF-2 at 85 °C in the absence or presence of 1 mM FA or 1 mM NaCl. The velocity of the reaction was significantly decreased by FA, whereas NaCl did not have any effect on activity. These findings suggest a specific effect of the antibiotic on the activity and exclude any interference due to the sodium ion from the antibiotic. It is worth mentioning that the inhibitory power of FA was not impaired under these experimental conditions, because incubation of FA at 85 °C for up to 30 min did not cause any effect on the inhibitory power exhibited by the preincubated antibiotic on the eubacterial EF-G functions.

To confirm the effect of FA on the rate of poly(Phe) synthesis, this reaction was carried out at 85 °C in the presence of increasing concentrations of FA. The velocity of the reaction, calculated from the linear kinetics, was reported as a function of the antibiotic concentration (Figure 2). A measurable decrease in *Ss*EF-2 activity was observed as the FA concentration increased. The effect of an antibiotic concentration higher than 1 mM was not measured because of the limited solubility of FA. The low level of inhibition exerted by FA on the *Ss*EF-2-dependent poly(Phe) synthesis has probably been overseen in other reports when the assay mixture contained a total extract of the macromolecular components from *S. solfataricus* (*19*). To confirm the responsiveness of *Ss*EF-2 to fusidic acid-like compounds, the effect of increasing concentrations of helvolic acid (HA) in the *Ss*EF-2-dependent poly(Phe) synthesis was also studied (Figure 2). The data indicate that this natural fusidane analogue of FA isolated from *Aspergillus fumigatus* is more effective than FA on the archaeal factor, a finding similar to that reported for the eubacterial EF-G (*5*).

The GTP breakdown catalyzed by *Ss*EF-2 represents a crucial step for the unidirectionality of the translocation process. This activity can be measured in two experimental systems uncoupled from translocation: the ribosome-dependent GTPase (GTPase^r; see ref *27*) or the intrinsic GTPase triggered in the presence of ethylene glycol and barium chloride (GTPase^g; see ref *28*). Both systems were used to study the effects of increasing concentrations of FA and HA. The inhibition profiles of GTPase^r obtained by the evaluation of the rate of this reaction at each indicated antibiotic concentration of FA and HA confirm the responsiveness of *Ss*EF-2 to these antibiotics (Figure 3A). Similar to what was found in poly(Phe) synthesis, HA was more effective than FA in inhibiting GTPase^r. When the effect of the antibiotics was checked in GTPase^g (Figure 3B), no inhibition by FA was revealed up to a concentration of 1 mM, whereas a low level of inhibition could be detected in the presence of the highest concentrations of HA.

Increased Sensitivity to Fusidanes in a Mutant SsEF-2 Carrying the L452R Amino Acid Replacement. In light of a possible increase in sensitivity of *Ss*EF-2 to fusidane-mediated inhibition, residue L452 was considered to be the target of a site-directed mutagenesis. In fact, in EF-G from *Salmonella typhimurium*, the corresponding R471 residue belongs to the putative antibiotic binding site. Furthermore, the *fusA* gene encoding EF-G isolated from a bacterial strain harboring FA resistance predicted the R471L amino acid replacement (*22*). Therefore, this position in the archaeal factor is occupied by an amino acid residue that causes FA resistance when present in the eubacterial EF-G. For this reason, we attempted to obtain a mutant form of *Ss*EF-2 containing the L452R substitution.

The functionality of L452R *Ss*EF-2 in the absence of an antibiotic was assessed by comparing its biochemical properties with those of wild-type *Ss*EF-2 (Table 1). A measurable reduction of the activity levels was observed for all the tested

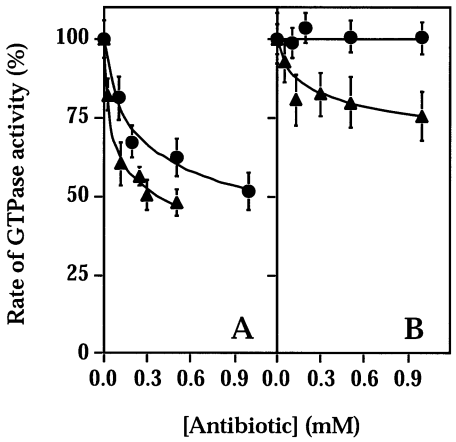

FIGURE 3: Effect of fusidane antibiotics on GTPase activities supported by *Ss*EF-2. (A) Kinetics of GTPase^r in the presence of the indicated concentrations of sodium fusidate (●) or sodium helvolate (▲) were performed in reaction mixtures containing, in a final volume of buffer C of 250 μL, 0.4 μM *S. solfataricus* ribosome and 0.1 μM *Ss*EF-2. The reaction was carried out at 85 °C and started with the addition of [γ-^{32}P]GTP up to a final concentration of 100 μM (specific activity, 110 cpm/pmol). After 15, 30, 45, and 60 s, 50 μL aliquots were withdrawn from the reaction mixture and analyzed for the amount of $^{32}P_i$ released as described in Materials and Methods. Blanks run in the absence of *Ss*EF-2 were run in parallel and subtracted. The rate values were calculated from the slope of linear kinetics and expressed as percentages of the rate calculated in the absence of antibiotic [0.53 mol of GTP hydrolyzed (mol of *Ss*EF-2)$^{-1}$ s^{-1}]. (B) Kinetics of GTPase^g in the presence of the indicated concentrations of sodium fusidate (●) or sodium helvolate (▲) were performed in reaction mixtures containing, in a final volume of buffer D of 250 μL, 0.2 μM *Ss*EF-2. The reaction was carried out at 85 °C and started with the addition of [γ-^{32}P]GTP up to a final concentration of 50 μM (specific activity, 110 cpm/pmol). After 2, 4, 6, and 8 min, 50 μL aliquots were withdrawn from the reaction mixture and analyzed for the amount of $^{32}P_i$ released as described in Materials and Methods. Blanks run in the absence of *Ss*EF-2 were run in parallel and subtracted. The rates were calculated from the slope of linear kinetics and expressed as percentages of the rate calculated in the absence of antibiotic [0.039 mol of GTP hydrolyzed (mol of *Ss*EF-2)$^{-1}$ s^{-1}].

Table 1: Effect of the L452R Amino Acid Replacement on the Biochemical Properties of *Ss*EF-2 Measured at 85 °C

	poly(Phe) synthesis [mol of Phe incorporated (mol of protein)$^{-1}$ s^{-1}]	GTPase^r [mol of GTP hydrolyzed (mol of protein)$^{-1}$ s^{-1}]	GTPase^g [mol of GTP hydrolyzed (mol of protein)$^{-1}$ s^{-1}]
*Ss*EF-2	0.011 ± 0.001	0.53 ± 0.04	0.039 ± 0.003
L452R *Ss*EF-2	0.0018 ± 0.0005	0.12 ± 0.01	0.015 ± 0.002

biochemical properties of the mutant. However, L452R *Ss*EF-2 is able to support poly(Phe) synthesis, GTPase^r and GTPase^g. This finding indicates that it was possible to measure the effects of antibiotics on any activity supported by the mutant.

The effect of FA and HA on the rate of poly(Phe) synthesis supported by L452R *Ss*EF-2 is shown in Figure 4. The comparison of the inhibition profiles of L452R *Ss*EF-2 with those previously described for wild-type *Ss*EF-2 points to an increased sensitivity of the mutant to the inhibition by each antibiotic, HA remaining more effective than FA. A similar behavior emerged from the inhibition profiles obtained from the GTPase^r system supported by L452R *Ss*EF-2 (Figure 5A). On the other hand, in the GTPase^g system (Figure 5B), the activity of L452R *Ss*EF-2 slightly was decreased even by FA, a feature not observed with the wild-type factor. As expected, this low responsiveness of the mutant further improved when the effect of HA was considered. These latter findings indicate a sensitivity of L452R *Ss*EF-2 to both fusidanes even in the absence of the ribosome.

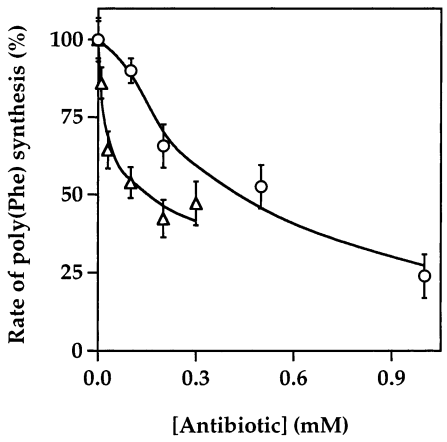

FIGURE 4: Effect of fusidane antibiotics on poly(Phe) synthesis supported by L452R *Ss*EF-2. The rate of poly(Phe) synthesis supported by L452R *Ss*EF-2 in the presence of the indicated concentrations of sodium fusidate (○) or sodium helvolate (△) was calculated from kinetics essentially performed as indicated in the legend of Figure 1, with the exception that L452R *Ss*EF-2 replaced *Ss*EF-2 and that the kinetics were extended up to 5 min. The rates were expressed as percentages of the rate calculated in the absence of antibiotic [0.0018 mol of Phe incorporated (mol of L452R *Ss*EF-2)$^{-1}$ s^{-1}].

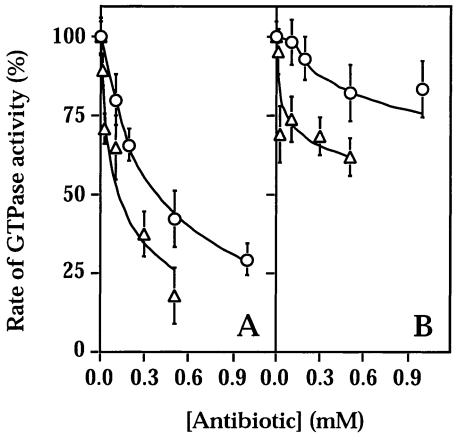

FIGURE 5: Effect of fusidane antibiotics on GTPase activities supported by L452R *Ss*EF-2. (A) Effect of the indicated concentrations of sodium fusidate (○) or sodium helvolate (△) on the rate of GTPase^r supported by L452R *Ss*EF-2. The experimental conditions were identical to those reported in the legend of Figure 3A, with the exception that L452R *Ss*EF-2 replaced *Ss*EF-2 and that the kinetics were extended up to 3 min. The rate calculated in the absence of antibiotic was 0.12 mol of GTP hydrolyzed (mol of *Ss*EF-2)$^{-1}$ s^{-1}. (B) Effect of the indicated concentrations of sodium fusidate (○) or sodium helvolate (△) on the rate of GTPase^g supported by L452R *Ss*EF-2. The experimental conditions were identical to those reported in the legend of Figure 3B, with the exception that L452R *Ss*EF-2 replaced *Ss*EF-2 and that the kinetics were extended up to 16 min. The rate calculated in the absence of antibiotic was 0.015 mol of GTP hydrolyzed (mol of *Ss*EF-2)$^{-1}$ s^{-1}.

Table 2: Concentration of Fusidic Acid and Helvolic Acid Leading to 50% Inhibition (IC_{50}) of the Biochemical Properties of *Ss*EF-2 and L452R *Ss*EF-2

		IC_{50}[a] (mM)	
	biochemical property	fusidic acid	helvolic acid
*Ss*EF-2	poly(Phe) synthesis	0.93 ± 0.06	0.57 ± 0.04
*Ss*EF-2	ribosome-dependent GTPase ($GTPase^r$)	1.1 ± 0.1	0.37 ± 0.03
*Ss*EF-2	intrinsic GTPase ($GTPase^g$)	none	$\gg 1$
L452R *Ss*EF-2	poly(Phe) synthesis	0.43 ± 0.03	0.15 ± 0.02
L452R *Ss*EF-2	ribosome-dependent GTPase ($GTPase^r$)	0.38 ± 0.03	0.12 ± 0.02
L452R *Ss*EF-2	intrinsic GTPase ($GTPase^g$)	$\gg 1$	> 1

[a] The values were calculated as described in Materials and Methods using the inhibition profiles reported from Figure 2 to Figure 5.

DISCUSSION

This paper describes the fusidane sensitivity of archaeal *Ss*EF-2. Our data show that the antibiotic FA is able to cause a partial impairment of the biochemical properties supported by *Ss*EF-2, such as poly(U)-directed poly(Phe) synthesis and ribosome-dependent GTPase. The effect of FA on the biochemical properties of *Ss*EF-2 is observed in the presence of the ribosome, as confirmed by the lack of responsiveness of the intrinsic GTPase system of *Ss*EF-2. In this case, FA does not cause any reduction in the activity in the range of antibiotic concentrations that was tested. These features resemble what was reported for *E. coli* EF-G. In that case, FA inhibited the ribosome-dependent GTPase of EF-G (*31*), but did not affect its intrinsic GTPase triggered by 2-propanol (*32*). On the basis of the significantly stronger inhibition observed in the eubacterial system (*5*), FA can be considered a weak inhibitor of the archaeal factor.

Other experiments proved that it is possible to increase the sensitivity of *Ss*EF-2 to fusidane inhibition. First, the antibiotic sensitivity of the archaeal factor depends on the structure of the fusidane. The natural fusidane analogue HA causes a higher level of impairment of the biochemical properties of *Ss*EF-2, and a similar finding was also realized with the eubacterial EF-G (*5*). Also, the L452R amino acid replacement in *Ss*EF-2 improves the sensitivity of the archaeal factor. In this respect, the L452 position of *Ss*EF-2 is particularly interesting, because it is known that the eubacterial EF-G became resistant to FA when its corresponding conserved arginine (R471 in *S. typhimurium* EF-G) was replaced with leucine (*22*). The relevance of this position in fusidane interaction is also confirmed by the three-dimensional structure of EF-G from *Thermus thermophilus* (*12*−*14*). In this case, the corresponding R465 residue is located in the poorly determined domain III, which is part of the putative antibiotic binding site together with domains I and V. Therefore, it is likely that the lower sensitivity to fusidane inhibition displayed by *Ss*EF-2 is in part related to the occurrence of a leucine residue in this position in place of the eubacterial arginine. It is interesting that even eukaryal EF-2 displays a leucine in this position (*21*).

The sensitivity to fusidanes of *Ss*EF-2 is shown in Table 2, wihch lists values of antibiotic concentration leading to 50% inhibition (IC_{50}) of the biochemical activities of wild-type and mutant *Ss*EF-2. In some cases, the IC_{50} value is either missing or roughly estimated, because of the absent or low antibiotic responsiveness emerging from the $GTPase^g$ system. The IC_{50} values depend on the binding affinity of the antibiotic and the maximum inhibitory effect at a saturating antibiotic concentration. However, because of the limited solubility of both antibiotics, the maximum inhibitory effect was difficult to determine, and therefore, the values listed in Table 2 could not represent the actual IC_{50} values. As a consequence, the complete inhibition of the translocation process was not achieved in *S. solfataricus*. This feature represents a significant difference from the eubacterial system, which is completely blocked in the presence of 1 mM FA (*5*). Nevertheless, the IC_{50} values are useful for a comparison of the effectiveness of FA and HA in different systems, even because the maximum solubility of both antibiotics is quite similar. Indeed, under all experimental conditions, the inhibition capacity of HA is higher than that exhibited by FA, the ratio of HA/FA effectiveness being in the range of 1.6−3.2. This behavior, consistently observed also in the $GTPase^g$ system and after the L452R replacement in *Ss*EF-2, might indicate that the HA structure is better adapted for the interaction between fusidane and the archaeal factor. On the other hand, the L452R replacement causes an increased sensitivity of *Ss*EF-2 to both fusidanes in all the biochemical assays, the stimulation brought about by the mutation ranging between 2.2- and 3.8-fold. The combined action of these two parameters affecting the antibiotic sensitivity of *Ss*EF-2 is mutually independent, and the responsiveness of the archaeal factor improved by nearly 1 order of magnitude when considering the combined effect of the best adapted structure of HA and the favorable L452R amino acid mutation. A final consideration emerging from the analysis of the data regarding the $GTPase^g$ systems suggests a possible direct interaction between the archaeal factor and fusidanes in the absence of the ribosome. In fact, a low level of impairment of intrinsic GTPase has been measured when considering the effects of either HA on *Ss*EF-2 or both fusidanes on L452R *Ss*EF-2. However, the ribosome remains an essential component in improving the antibiotic sensitivity of the archaeal factor.

In conclusion, this paper shows that the archaeal *Ss*EF-2 is responsive to fusidane inhibition. A focus on the parameters affecting the antibiotic sensitivity of *Ss*EF-2 pointed to the relevance of three main effectors: the presence of the ribosome, an adapted fusidane structure, and a specific amino acid replacement. However, the difference in sensitivity between wild-type and mutant *Ss*EF-2 will be better evaluated when other mutants are available. All these studies indicate that the structural requirements for fusidane inhibition are also likely conserved in *S. solfataricus*. To understand how the structural differences between members of the same antibiotic family or specific amino acid replacements affect the properties of *Ss*EF-2, these findings should be reinterpreted when structural information about the archaeal *Ss*EF-2 is available.

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